Highly functionalized $\beta$-lactams and 2-ketopiperazines as TRPM8 antagonists with antiallodynic activity

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The cool sensor transient receptor potential melastatin channel 8 (TRPM8) is highly expressed in trigeminal and dorsal root ganglia, playing a key role in cold hypersensitivity associated to different peripheral neuropathies. Moreover, these channels are aberrantly expressed in different cancers, and seem to participate in tumor progression, survival and invasion. Accordingly, the search for potent and selective TRPM8 modulators attracted great interest in recent years. We describe new heterocyclic TRPM8 antagonist chemotypes derived from N-chloroalkyl phenylalaninol-Phe conjugates. The cyclization of these conjugates afforded highly substituted $\beta$-lactams and/or 2-ketopiperazine (KP) derivatives, with regioselectivity depending on the N-chloroalkyl group and the configuration. These derivatives behave as TRPM8 antagonists in the Ca²⁺ microfluorometry assay, and confirmed electrophysiologically for the best enantiopure $\beta$-lactams 24a and 29a (IC₅₀, 1.4 and 0.8 µM).

Two putative binding sites by the pore zone, different from those found for typical agonists and antagonists, were identified by in silico studies for both $\beta$-lactams and KPs. $\beta$-Lactams 24a and 29a display antitumor activity in different human tumor cell lines (micromolar potencies, A549, HT29, PSN1), but correlation with TRPM8 expression could not be established. Additionally, compound 24a significantly reduced cold allodynia in a mice model of oxaliplatin-induced peripheral neuropathy.

The transient receptor potential melastatin 8 (TRPM8) receptor is a multimodal channel, activated by cold and cooling compounds, such as menthol and icilin, but also by membrane depolarization and changes in extracellular osmolarity¹. In the periphery, these channels are highly expressed in the afferent Aδ and C fibers of sensory neurons, where they have been implicated in the perception and transduction of pain. Thus, cumulative evidence is signaling TRPM8 channels as pivotal players in cold hypersensitivity, especially that provoked by cancer chemotherapy²-⁴. Acute and chronic oxaliplatin-induced cold hypersensitivity has been reproduced in rats and correlated with TRPM8 expression and function⁵,⁶. Similarly, it is known that TRPM8 channels are implicated in inflammatory pain and migraine⁷. The thesis that migraine locus lies in the peripheral nervous system⁸, and the identification of TRPM8 as a candidate susceptibility gene for migraine⁹,¹⁰, points to the modulation of TRPM8 channels as a plausible mechanism for the treatment of this pathology¹¹.

TRPM8 channels are also expressed in prostate, pancreas, and vascular, bronchopulmonary and urogenital tissues¹²-¹⁶. There are also numerous experimental evidences demonstrating that TRPM8 channels play important roles in tumor development and progression, including prostate, pancreas, breast, lung, colon, bladder and melanoma malignancies, among others¹²,¹⁷. In many cases, the aberrant expression of TRPM8 channels is correlated to tumor growth, progression and invasion capacity, at least in initial stages¹⁸-⁲¹, while sometimes TRPM8 channels are downregulated in final phases of the disease, and their activation seems to have a protective role²².

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It is also described that TRPM8 is expressed in the central nervous system, thus opening new opportunities to study and understand its potential role within the brain.

Cryo-electron microscopy has demonstrated that the TRPM8 channel is a tetrameric protein, with 6 TM helical segments (S1-S6) and intracellular N- and C-terminal domains, with the pore delimited by a region situated between S5 and S6 helices. The structure of TRPM8 in complex with some agonist compounds is disclosed, indicating a cavity for agonists delimited by S1, S4 and the TRP domain. This highly adaptable pocket, delineated by the lower part of the S1–S4 transmembrane and the TRP domain, has also been described as the main cleft to bind AMTB and CT-I antagonists.

Because of the physiological and therapeutic significance of TRPM8 channels, many efforts have been devoted to the search for selective modulators, both agonists and antagonists. Among the agonists, we can found menthol, diverse menthol derivatives, icilin, tertiary amides and different natural products. As for the antagonists, most important chemotypes encompass different heterocyclic systems, including monocyclic central cores, like thiazole and β-lactams, and bicyclic structures, as benzothiophene, benzimidazole, and isoquinoline, among others. Also different acyclic central scaffolds (amide, sulfonamide, urea, glycine, tryptophan), decorated with aromatic and heterocyclic rings are reported as TRPM8 antagonists (Fig. 1). Among the latter, AMG-333 and PF-05105679 reached phase I clinical trials for the oral treatment of cold induced pain and migraine.
respectively\textsuperscript{33,36}, but both studies were discontinued due to adverse secondary effects, including non-tolerated hot sensations. Therefore, there is still a need for TRPM8 antagonists with improved properties.

In this context, we have recently described a series of compounds derived from Phe and Asp/Glu amino acid conjugates and having a monocyclic \( \beta \)-lactam central core, which were able to potently and selectively inhibit the activation of TRPM8 by menthol, cool and voltage\textsuperscript{37}. Among this series, the shorter Asp derivative 1 (n = 2, Fig. 1) was more potent than the longer Glu analogue 2 (n = 3, Fig. 1), while all the three benzyl and the Boc hydrophobic moieties are important for activity\textsuperscript{37}.

Looking for shorter \( \beta \)-lactam derivatives, bearing four hydrophobic substituents, in this manuscript we describe the preparation of conjugates of Z-phenylalaninol with amino acid derivatives and their cyclization to heterocyclic compounds having a \( \beta \)-lactam or a 2-ketopiperazine central scaffold. Both series of compounds behave as TRPM8 antagonists and, among them, selected \( \beta \)-lactam derivatives display antitumor activity, and antiallodynic properties in a model of chemotherapy-induced cold allodynia.

Results

Design. The preparation of a shorter analogue of compounds 1 and 2 was initially projected starting from Boc-Ser-OBn (n = 1). However, all attempts to condense this Ser derivative with Ns-Phe-OBn, were unsuccessful due to the formation of the corresponding dehydroalanine analogue, as described in related reactions\textsuperscript{38}. As the four-membered ring was higher when \( \text{Cs}_2\text{CO}_3 \) was used as base (Table 2). Similarly, the basic treatment of the C3, C4 dictated by the configuration of the chloropropionic moiety\textsuperscript{43}. In good agreement with our precedents, demonstrated that related 2-chloropropanoyl derivatives afforded pure \( \beta \)-lactams, with the stereochemistry at C3, C4 dictated by the configuration of the chloropropionic moiety\textsuperscript{43}. In good agreement with our precedents, the configuration was assigned as 4S.

To attempt to obtain \( \beta \)-lactams as single isomers, we prepare enantiopure 2-chloropropanoyl Z-phenylalaninol-Phe-OBn conjugates 22, 23, 27 and 28 (Scheme 2). For this, conjugates 8 and 9 were reacted with 2S- or 2R-chloropropionic acid in the presence of trichloroacetonitrile and triphenylphosphine. We have previously demonstrated that related 2-chloropropanoyl derivatives afforded pure \( \beta \)-lactams, with the stereochemistry at C3, C4 dictated by the configuration of the chloropropionic moiety\textsuperscript{43}. In good agreement with our precedents, the configuration was assigned as 4S.

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TRPM8 in vitro activity. The ability to inhibit menthol-induced Ca\textsuperscript{2+} intracellular influx into the cytosol on HEK293 cells heterologously expressing the rat TRPM8 channel was measured and compared to that of AMTB,
Scheme 1. Preparation of chloroacetyl phenylalaninol-Phe conjugates and cyclization reactions.
(+120 mV) was observed. A similar behaviour was detected for diastereomeric β-lactam derivatives (Fig. S4). The IC50 values were 1.4 ± 1.1 µM for 10ab (Fig. 2B) and 0.8 ± 1.1 µM for 29a (Fig. 2D).

Table 1. Results of the cyclization reaction of chloroacetyl phenylalaninol-Phe conjugates. NI not isolated.

| Starting Compd | Base | Time (h) | β-L/KP ratio (HPLC) | β-Lactam# Yield (%) | Configuration (isomer ratio) | KP # Yield (%) | Configuration (isomer ratio) |
|----------------|------|----------|---------------------|---------------------|-----------------------------|----------------|-----------------------------|
| 10             | BTPP | 5        | 3:97 NI             | –                   | 13ab (77)                  | 5S,1R/5S,1R (80:20) |
|                | BEMP | 3        | 2:98 NI             | –                   | NI                          | 5S,1R/5S,1R (93:7)  |
|                | Cs2CO3 | 3       | 11:89 12ab (8.5)   | 4S,2'S/4R,2'S (83:17)| 13ab (68)                  | 5S,1R/5S,1R (95:5)  |
| 11             | BTPP | 5        | 2:98 NI             | –                   | 15ab (77)                  | 5R,1S/5R,1R (81:19) |
|                | BEMP | 6        | 2:98 NI             | –                   | NI                          | 5R,1S/5R,1R (80:20) |
|                | Cs2CO3 | 6       | 27:73 14ab (11)    | 4S,2'R/4R,2'R (88:12)| 15ab (69)                  | 5R,1S/5R,1R (90:10) |
| 19             | BTPP | 5        | 0:100 –             | –                   | NI                          | 5S,1R/5S,1R (40:60) |
|                | Cs2CO3 | 6      | 0:100 –             | –                   | 20ab (60)                  | 5S,1R/5S,1R (4:96)  |
|                | –     | –        | –                   | –                   | 21ab (55)                  | 5S,1R/5S,1R (10:90) |
| 36             | BTPP | 5        | 0:100 –             | –                   | 38ab (81)                  | 5R,1S/5R,1R (86:14) |

a well-known TRPM8 antagonist. The results obtained for β-lactam and KP derivatives are depicted in Table 3. Representative recordings of fluorescence obtained in microfluorometry experiments for selected compounds are in Supplementary Fig. S3. No agonist activity was observed for these compounds in the absence of menthol.

As shown in Table 3, slightly better antagonist activity was observed for β-lactam with an N-2′-R-appendage (compare 2′R-derivatives 14ab, 29a and 31ab to 2′S-analogues 12ab, 24a and 25ab, respectively). However, in the case of diastereoisomeric mixtures, the exact contribution to the activity of each individual isomer cannot be assessed. As previously described for the first generation of β-lactam TRPM8 antagonists, the phenyl group at position 4 is important for Ca2+ entrance inhibition, since the 4-CH3 derivative 39a was one order of magnitude less active than the 4-Bn analogue 29a.

All KP derivatives were assayed as mixtures of two diastereoisomers, therefore the structure–activity relationships should be considered as tendencies, not as absolute statements. As for the β-lactam derivatives, the configuration of the stereocenter coming from the phenylalaninol moiety seems to dictate the antagonist activity, with 5R-KPs more potent than 5S-isomers (compare 15ab to 13ab) (Table 3). The 1′-configuration plays a role for the inhibition of menthol-induced TRPM8 activation, with a preference for the 1′R-configuration (in 21ab, 10:90) over the 1′S-isomer (13ab, 80:20). The OMe group in 21ab could participate in the direct interaction with the TRPM8 channel, as the corresponding OMe analogue 20ab shows an important drop in activity. In the 3-methyl derivatives, a 3R,5R configuration (in 30ab) is preferred over the 3S,5S combination (in 26ab), while the 3S,5R-configured diastereoisomers (32ab) showed the lowest activity in this series. In this case, a 4-Ch1 group led to slightly less active derivatives (38ab, 40ab) compared to the corresponding 4-Bn analogues (15ab and 30ab, respectively), although the fall in activity due to this modification is less acute than in the β-lactam series.

β-Lactam and 2-ketopiperazine derivatives were also assayed for their activity in cell expressing hTRPV1 channels. No significant antagonist activity was measured for any derivatives within both chemotypes (Supplementary Tables S1, S2, and Fig. S3), indicating their selectivity for TRPM8 channels.

The TRPM8 antagonist activity of the enantiopure β-lactams 24a and 29a was further confirmed electrophysiologically by Patch-clamp experiments, using the whole cell configuration in HEK293 cells expressing TRPM8 channels.

As shown in Fig. 2, perfusion with 100 µM menthol gives rise to a strongly outward rectifying ionic current characterized by the presence of negligible current at negative potential and the presence of a linear current increase (ohmic) at positive voltages ≥ 40 mV (1–50 mV/I + 120 mV = 0.07). When 10 µM of 24a was applied (Fig. 2A, blue), an important reduction on the menthol-mediated TRPM8 activity at depolarizing voltages (+120 mV) was observed. A similar behaviour was detected for diastereomeric β-lactam 29a (Fig. 2C, blue). The dose–response curve for both compounds was obtained at a holding potential of -60 mV (Supplementary Fig. S4). The IC50 Values were 1.4 ± 1.1 µM for 24a (Fig. 2B) and 0.8 ± 1.1 µM for 29a (Fig. 2D).

Docking studies. In order to investigate possible binding pockets within the TRPM8 channel for these families of KP and β-lactam TRPM8 antagonists, we performed computational studies with compounds 13a, 24a, and 29a. A model of the rat TRPM8 channel, created from the cryo-electron microscopy structure of the Ficedula albicollis (PDB code 6BPQ)24, was used, and docking simulations were performed with the software implemented in Yasara.44–46 These docking studies predicted that the three compounds most likely (> 80% solutions) interact with the TRPM8 by the pore zone, with two major solutions having the best binding energies (Supplementary Fig. S5, Table S3). Site 1 was identified in the middle of the transmembrane region, mainly involving TM5 (S5) and
Scheme 2. Preparation of 2-chloropropanoyl phenylalaninol-Phe conjugates and cyclization reactions.
mainly hydrophobic interactions can be distinguished at both binding pockets, with some π–π stacking and a loop connecting TM6 and TRP domains of the 4 protein subunits forming the channel. As for the compounds, TM6 (S6) of one monomer and segments of an adjacent subunit (S5 or S6 and/or the S5-S6 segment forming the four channel subunits (see Supplementary Fig. S7).

Table 2. Results of the cyclization reaction of 2-cloropropanoyl phenylalaninol conjugates. NI: not isolated.

| Compd | Isomers | Isomers ratio | % Blockade 50 μM | % Blockade 5 μM | IC50 (μM) |
|-------|---------|---------------|------------------|-----------------|-----------|
| β-Lactams |         |               |                  |                 |           |
| 12ab  | 4S,2'R/4R,2'S | 83:17 | 100.0 ± 3.1 | 92.3 ± 6.2 | 1.5 ± 1.2 |
| 14ab  | 4S,2'R/4R,2'R | 88:12 | 103 ± 2.7 | 91.0 ± 4.3 | 1.2 ± 1.0 |
| 24a   | 3S,4S,2'S | – | 110.7 ± 11.7 | 65.4 ± 6.4 | 2.4 ± 1.2 |
| 25ab  | 3R,4R,2'S/3S,4R,2'S | 85:15 | 89.2 ± 9.2 | 68.2 ± 5.6 | 3.9 ± 1.9 |
| 29a   | 3S,4S,2'R | – | 100.0 ± 5.0 | 97.2 ± 4.8 | 0.4 ± 1.5 |
| 31ab  | 3S,4R,2'R/3S,4R,2'R | 77:23 | 105.0 ± 5.5 | 91.0 ± 4.3 | 1.6 ± 1.3 |
| 39a   | 3S,4S,2'R | – | 100.4 ± 3.5 | 46.6 ± 11.1 | 6.2 ± 1.1 |
| 2-Ketopiperazines |         |               |                  |                 |           |
| 13ab  | 5S,1'S/5S,1'R | 80:20 | 102.1 ± 7.7 | 48.9 ± 6.2 | 17.9 ± 1.3 |
| 15ab  | 5R,1'S/5S,1'R | 81:19 | 106 ± 5.06 | 101.4 ± 6.1 | 1.8 ± 1.9 |
| 20a   | 5S,1'S/5S,1'R | 4:96 | 610 ± 5.6 | 223 ± 5.5 | 18.8 ± 1.7 |
| 21a   | 5S,1'S/5S,1'R | 10:90 | 92.5 ± 4.3 | 70.0 ± 6.8 | 2.0 ± 1.5 |
| 26ab  | 3S,5S,1'S/3S,5S,1'R | 81:19 | 102.4 ± 15 | 93.5 ± 3.4 | 2.4 ± 1.3 |
| 30ab  | 3R,5R,1'S/3S,5R,1'R | 82:18 | 101.2 ± 11.4 | 101.7 ± 5.3 | 0.16 ± 1.6 |
| 32ab  | 3S,5R,1'S/3S,5R,1'R | 96:4 | 115.1 ± 4.5 | 47.4 ± 6.8 | 17.4 ± 1.6 |
| 38ab  | 5R,5R,1'S/1'R | 86:14 | 105 ± 3.2 | 66.0 ± 3.3 | 2.5 ± 1.3 |
| 40ab  | 3S,5R,1'S/3S,5R,1'R | 58:42 | 107.9 ± 2.5 | 79.1 ± 3.3 | 0.8 ± 1.2 |
| AMTB  | – | – | – | – | 7.3 ± 1.5 |

Table 3. Activity at TRPM8 of β-lactams derived from phenylalaninol conjugates.

TM6 (S6) of one monomer and segments of an adjacent subunit (S5 or S6 and/or the S5-S6 segment forming the pore). The second binding compartment, Site 2, correspond to the cytosolic mouth of the pore, involving the loops connecting TM6 and TRP of the 4 protein subunits forming the channel. As for the compounds, mainly hydrophobic interactions can be distinguished at both binding pockets, with some π–π stacking and a few H-bonds identified.

Ketopiperazine 13a binds TRPM8 channel at Site 1 through two π–π stacking interactions, a face-to-face stacked interaction between the phenyl group of the 1'-Bz moiety and Y963 at subunit 1 (S6), and secondly a T-shaped (edge-to-face) contact encompassing the phenyl group of the α-CO2-Bz moiety and F874 residue of TRPM8 subunit 3 (S5) (Supplementary Fig. S6). Among hydrophobic interactions, the Ph group of the Cbz moiety occupies a hydrophobic pocket delineated by residues located at subunit 3 (F870, L873, F874, I962, L965, and I969) and subunit 2 (I844). In addition, the 1'-Bn moiety is also involved in hydrophobic interactions through L871 and F874 (Subunit 3), while the phenyl group of the 5'-Bn is in touch with I962 and Y963, also at subunit 3. At Site 2, a face-to-face π–π stacking involves the Ph group of the Cbz and the aromatic ring of the Y981 residue (subunit 1). Moreover, the hydrophobic interactions comprise the four Ph rings of 13a and residues from the four channel subunits (see Supplementary Fig. S7).

At Site 1, β-Lactams 24a and 29a occupy similar areas of the transmembrane region, involving two neighboring channel subunits, but their poses are clearly different, with the β-lactam ring pointing to the upper and
lower part of the binding pocket, respectively (Fig. 3 and Supplementary Figs. S8–S11). The 3,4,2′S isomer 24a binds the channel through a H-bond involving its NH and the backbone CO of L959 (S5, subunit 3) (Fig. 4 and Supplementary Fig. S8). Additionally, three π–π interactions contribute to the complex stabilization, two T-shaped contacts comprising the Ph group of the Cbz moiety and W877 and F881 side-chains (both at S5), and a face-to-face sandwich between the aromatic groups of 2′-Bn and Y963 residue (S6), all at channel monomer 3. This monomer contributes also to the complex with nine hydrophobic interactions among a series of residue side-chains of the channel and the four Ph groups of the antagonist, while subunit 2 (S5 and S5-S6 loop) add three additional hydrophobic contacts (specified in Supplementary Fig. S8). The diastereoisomeric 3,4,2′R compounds 29a is fixed to the channel through a H-bond (NH → CO of G913, S5-S6 segment, subunit 1) and a parallel displaced π–π connection (OBn-Y963 of S6, subunit 1, Supplementary Fig. S10). Also, a number of residues at subunit 1 provide hydrophobic interactions with the four Ph groups of 29a. As previously, the contiguous monomer (subunit 4) also supplies additional stabilizing interactions, in this case involving three residues at the pore-forming S5-S6 segment (Supplementary Fig. S10).

Main residues involved in the hydrophobic interactions of 24a and 29a at Site 2 are Y981 (from three out of four subunits), T982 of monomer 4, and I985 of two subunits. In addition, a H-bond between the CO group of Y981 (subunit 1, S6) and the NH group of 24a, and a π–π displaced stacking interaction involving the phenyl groups of the 2′-Bn moiety in 29a and Y981 side-chain (Subunit 1, S6), contribute to the respective stabilization of the complexes at Site 2 (Fig. 4, and Supplementary Figs. S9 and S11). Interestingly, most residues of rTRPM8 suggested as important for the interaction with β-lactams and KPs at both sites 1 and 2 are highly conserved in hTRPM8 (Supplementary Fig. S12).

Growth inhibitory activity in tumor cells. A number of recent experimental evidences position TRPM channels as important players in cancer growth and progression. Among these channels, the aberrant expression of the TRPM8 subtype has been described in different human malignant tumors, including those of prostate, pancreas, breast, colon, and skin, among others. More importantly, sometimes the TRPM8 overexpression was associated to poor prognosis of cancer patients. In good agreement, several, structurally different TRPM8 antagonists demonstrated good antitumor activity in prostate, and others human tumor cell lines. A few
years ago we have described that Phe-derived simpler β-lactams showed antitumor activity against three human cancer cell lines. Based on these precedents, in this work we first aimed at evaluating the antitumor activity of enantiopure compounds 24a and 29a. Initially, we assessed the growth inhibition percentage by a 10 µM concentration of these compounds on a 60 tumor cell screen at the National Cancer Institute (USA). In general, both β-lactam derivatives showed non-selective, modest cytotoxic effects in all cell assays, with the best data for leukemia (MOLT-4 cell line), melanoma (SK-MEL-5), lung (A549), colon (COLO-5), ovarian (OVCAR-4), renal (A498) and prostate (PC-3) cancer lines. We then measured the in vitro cytotoxic activity of 24a and 29a in four human tumor cell lines, namely A459 (lung), HT29 (colon), MDA-MB-231 (breast) and PSN1 (pancreas). The results are recorded in Tables 4 and S4, and compared to those of the well-known chemotherapeutic agent doxorubicin. As shown in Table 4, compound 24a displays in vitro cytotoxic activity in the micromolar range in three out of four assayed tumor cell lines, with no activity against the MDA-MB-231 breast cell line (at 10 µM/mL). Compared to 24a, slightly lower potencies were measured for β-lactam 29a in lung, colon and pancreas tumor cell lines, but contrasting it displays better, although moderate, in vitro cytotoxic activity in the breast cell line. Hence, no significant influence neither of the configuration of the β-lactam derivative nor of the TRPM8 antagonist potency was observed on the antiproliferative activity of these compounds. In general, these activities were one order of magnitude less potent than the control doxorubicin. Three steroisomeric 2-KP derivatives, having different TRPM8 antagonist capacity, were also assayed for their antitumoral activity (Table 4). In this case, compound 30ab, showing submicromolar TRPM8 antagonist activity, is only moderately active in A549 and PSN1 cell lines, with no significant cytotoxicity in the colon and breast cell lines. Compound 15ab, with micromolar potency as TRPM8 antagonist, is only cytotoxic in the pancreas cell line, while the less potent analogue 13ab did not show any significant antiproliferative activity. It seems that for the 2-KP series the in vitro cytotoxicity follows the same order than TRPM8 antagonist potency. The lower antitumor potential, compared to β-lactams, could be due either to the evaluation of diasteroisomeric mixtures in 2-KPs versus enantiopure β-lactams, or to the fact that the cytotoxicity of β-lactam derivatives is independent of the TRPM8 activity or both.

Figure 3. Low energy binding sites for β-lactam derivative 24a, Site 1 (A), Site 2 (B). Compound 24a is in pale orange, while side-chains of TRPM8 involved in the interaction are depicted in blue and labelled. Heteroatoms are indicated in red (O) and dark blue (N). H atoms have been removed.
The expression of TRPM8 channels in different cancer cell lines has been scarcely studied. To the best of our knowledge, there are no data on expression levels in A549, HT29 and PSN1 tumor cell lines, while for MDA-MB-231 the results described so far are contradictory. Thus, while the TRPM8 antagonist AMTB decreases viable cells in MDA-MB-231 breast cancer cells and TRPM8 levels are high in basal breast cancers, the TRPM8 expression does not seem very high in this and other breast cancer cell lines. On the contrary, a work by Liu and coworkers identified high levels of channel expression in different breast cancer cell lines, including MDA-MB-231. Because this lack of information and the controversial results, we cannot assure that the antitumor activity displayed by our compounds is partially due to a high expression of TRPM8 in the indicated cell lines or if it could be an effect totally independent of these channels.

No apparent, significant cytotoxic effects were observed for the β-lactam derivatives in HEK293 cells (up to 500 µM concentration, MTT assay).

**Antiallodynic effects in vivo.** Cold allodynia (painful sensation at cold temperatures that do not usually cause pain) and cold hyperalgesia (increased sensitivity to distressing cold temperatures) are associated to different peripheral neuropathies. Several chemotherapeutic agents in first clinical line induce peripheral neuropathies (known as CIPN), affecting million patients worldwide and limiting the dose administered to them, as well as the quality of life of many survivors. In oxaliplatin CIPN, the increased sensitivity to cold has been correlated to an augmented expression of TRPM8 channels, among others. In good correlation, there are recent experimental evidences describing that TRPM8 antagonists are able to decrease oxaliplatin-induced allodynia and cold hypersensitivity. According to these discoveries, we decided to explore the effects of β-lactam derivative 24a in an in vivo model of oxaliplatin CIPN, using acetone assay for monitoring cold allodynia. In male mice, the injection of oxaliplatin on days 1, 3 and 5 at a 6 mg/kg dose produces peripheral cold allodynia. As shown in Fig. 4, the intraplantar (i.pl.) administration of β-lactam derivative 24a (1 µg, A or 3 µg/ipl, B) was administered to the oxaliplatin treated animals, and the time-course of cold allodynia was measured. Data are given ± SME (n = 5). **P < 0.05; ***P < 0.001; ****P < 0.0001.

### Table 4. In vitro cytotoxicity (GI50, µM) of compounds 24a and 29a on four human cell cancer lines.

| Compd. | Family | TRPM8 IC50 (µM) | Lung-NSCLC | Colon | Breast | Pancreas |
|--------|--------|-----------------|------------|-------|--------|---------|
| 24a    | β-Lactam | 2.4 ± 1.2       | 3.29       | 4.16  | > 17.3 | 5.55    |
| 29a    | β-Lactam | 0.4 ± 1.5       | 5.90       | 7.12  | 12.7   | 6.42    |
| 13ab   | 2-KP    | 17.9 ± 1.3      | > 17.8     | > 17.8| > 17.8 | > 17.8  |
| 15ab   | 2-KP    | 1.8 ± 1.9       | > 17.8     | > 17.8| > 17.8 | 6.4     |
| 30ab   | 2-KP    | 0.16 ± 1.6      | 12.7       | > 17.3| > 17.3 | 8.3     |
| Doxorubicin.HCl | –     | 0.24            | 0.19       | 0.17  | 0.17   | 0.17    |

**Discussion**

To search for new TRPM8 antagonist chemotypes, we explore the base-assisted cyclization of linear phenyllaninol-Phe conjugates, which afforded chiral β-lactam and/or 2-ketopiperazine (KP) heterocyclic derivatives. The regioselectivity (β-lactam versus KP) was dependent on the chloroalkyl substituent and the configuration of the linear precursor. While 2-chloroacetyl derivatives gave almost exclusively to the KP six-membered ring heterocycle, the cyclization of 2-chloropropanoyl analogues is governed by the configuration of both the...
phenylalaninol-derived (2′) and the 2-chloropropanoyl (2″) stereocenters. In short, 2′S,2″S and 2′R,2″R isomers provide β-lactams as the very major component of the reaction, while the KP heterocycle predominates after the cyclization of the 2′S,2″S R diastereoisomer, and the 2′S,2″R linear precursor provides almost the same amount of the four- and six-membered heterocyclic systems. The epimerization at the C-3 stereocenter in 3-methyl-β-lactam derivatives, not previously observed for related 2-azetidinones, was low for 3S,4S-configured compounds and more important for 3R,4R-analogues.

Both, the phenylalaninol-Phe-derived β-lactams and KPs behave as new TRPM8 antagonist chemotypes, blocking the channel activation by menthol (Ca2+ entry assay) with micromolar or submicromolar potencies, and did not show activity at hTRPV1. Single isomer β-lactams 24a and 29a display IC50 values of 2.4 and 0.4 µM, respectively, indicating that a 2′R-configuration of the phenylalaninol-derived substituent is preferred for TRPM8 antagonist activity. These antagonist activities were further confirmed using electrophysiology experiments, with Patch-clamp measurements sustaining that the 2′R diastereoisomer is slightly more potent than the corresponding 2′S isomer. In general, these phenylalaninol-Phe-derived β-lactams maintain significant TRPM8 blockade activity, although they showed somewhat decreased potency compared to the longer Asp-3-Phe analogues. For KPs, a 1′R- and a 5′R-configuration seem to favor the inhibition of TRPM8 channel activation.

Docking studies, using a homology model of rat TRPM8 channel, built on the cryo-electron microscopy structure of the TRPM8 from *Ficedula albicollis*27, propose two putative binding sites, by the pore zone, for the phenylalaninol-Phe-derived heterocyclic compounds described here. The first site involves transmembrane S5 and S6 of one channel subunit and the S5 or S6 and/or the S5–S6 segment forming the pore of one adjacent monomer, suggesting an allosteric modulation of the channel. The second most probable binding point is located at the bottom part of the pore, involving mainly hydrophobic interaction among the phenyl rings of the molecules and hydrophilic and aromatic residues of the four channel subunits, with the compound acting as a channel blocker. The sites predicted by these models of interaction differs from those proposed for tryptophan-derived antagonists33, and that from AMTB and TC-I/TRPM8 complexes solved by cryo-electron microscopy,28 which adopt different poses within the channel, but all around the menthol-binding pocket (delineated by the lower half of the TM4-TM5 helices and the TRP domain)25. The larger volume of our molecules could be behind this different behaviour.

TRPM8 channels are overexpressed in a number of tumors, like prostate, melanoma, lung and colon adenocarcinomes, and some TRPM8 antagonists demonstrated good antitumor activity32,40,46. Interestingly, enantiopure β-lactams 24a and 29a exhibited non-selective antitumor activity in different tumor cell lines, showing micromolar potency in four of them, while 2-KP regioisomeric compounds displayed lower antiflollowative activity. No direct correlation between TRPM8 antagonist and antitumor activity could be established. Abnormalities in TRPM8 expression was also found in models of chemotherapy-induced peripheral neuropathy57. To evaluate β-lactams and/or 2-ketopiperazine, allow the identification of new hits for TRPM8 modulation. Therefore, these two new chemotypes could constitute the starting point for further modifications on the road to improved compounds for future therapeutic applications in both pain and cancer.

**Experimental section**

**Chemistry.** Preparation of synthetic intermediates, their characterization and that of most final compounds are detailed in Supplementary Information.

**Cyclization reactions.** BTPP (2.4 mmol, 0.75 mL), or BEMP (2.4 mmol, 0.69 mL) or Cs2CO3 (3.2 mmol, 1.04 g) was added to a solution of the corresponding N-alkyl-N-chloroacetetyl- or N-alkyl-N-chloropropanoyl-Xaa derivative (1.6 mmol) in dry CH3CN (4 mL), under Ar atmosphere. The reaction mixture was stirred until consumption of the starting material. Then, the solvent was removed and the residue was extracted with EtOAc, filtered, and concentrated. The resulting residue was purified by flash chromatography on silica gel, using the eluent indicated in each case.

**4S-Benzyl-4-benzyloxy carbonyl-3S-methyl-1-[(2′S-benzyloxy carbonylamino-3′-phenyl) prop-1′-y1]-2-oxazetidine (24a).** Syrup. Yield: 65% (from 22, B: BTPP), Eluent: EtOAc:Hxene (1:2). HPLC: tR = 16.20 min (gradient of 30% to 95% of A, in 20 min). [α]D = −35.04 (c 1, CHCl3). Isomer ratio M(3S,2′S):m(3R,4S,2′S) = 97:3. 1H NMR (400 MHz, CDCl3): δ 7.35–6.96 (m, 20H, Ar), 5.75 (d, 1H, J = 14.5 Hz, 4-CH2), 3.23 (dd, 1H, J = 14.5, 8.0 Hz, 3′J = 14.0, 8.0 Hz, 3″-CH3), 3.00 (dd, 1H, J = 14.5, 4.0 Hz, 1″-CH3), 2.86 (dd, 1H, J = 14.0, 8.0 Hz, 3–H), 2.72 (dd, 1H, J = 14.0, 7.0 Hz, 3′–H), 1.08 (d, 3H, J = 7.5 Hz, 3-CH3). 13C NMR (75 MHz, CDCl3): δ 171.0 (COO), 170.6 (C2), 156.0 (OCON), 137.8, 137.0, 134.9, 134.8, 129.7, 129.2, 128.9, 128.8, 128.7, 128.5, 128.4, 127.9, 127.8, 127.5, 126.5 (Ar).
4S-Benzyl-4-benzyloxycarbonyl-3S-methyl-1-[(2'R-benzyloxycarbonylamino-3'-phenyl)prop-1'-yl]-2-oxoazetidine (29a). Syrup. Yield: 39% (from 27; B: BTFF). Eluent: EtOAc:Hexane (1:3). HPLC: t_R = 16.36 min (gradient of 30% to 95% of A, in 20 min). [α]_D = -72.67 (c 1, CHCl₃). Isomer ratio M(3S,4S,2'R):m(3R,4S,2'R) = 97.5:2.5. ¹H NMR (400 MHz, CDCl₃): δ 7.37–6.99 (m, 20H, Ar), 5.83 (d, 1H, J = 8.1 Hz, 2-NH), 5.27 (d, 1H, J = 12.0 Hz, OCH₂), 5.17 (d, 1H, J = 12.0 Hz, OCH₂), 5.05 (s, 2H, OCH₂), 4.07 (m, 1H, 2'-H), 3.56 (d, 1H, J = 14.5 Hz, 4-CH₂), 3.43 (q, 1H, J = 7.6 Hz, 3-1H), 3.03 (d, 1H, J = 14.5 Hz, 4-CH₂), 2.99 (m, 2H, 1'-H), 2.71 (dd, 1H, J = 13.5, 7.4 Hz, 3'-H), 2.64 (dd, 1H, J = 13.4, 6.6 Hz, 3'-H), 1.08 (dd, 3H, J = 7.5 Hz, 3-CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 171.3 (COO), 170.3 (C₂), 156.3 (OCON), 137.5, 135.1, 134.8, 129.7, 129.5, 129.2, 129.0, 128.9, 128.8, 128.7, 128.5, 127.9, 126.5 (Ar), 69.0 (C₄), 67.8, 66.3 (OCH₂), 54.1 (C₃), 51.6 (C₂) (2'), 47.0 (C₁) (2'), 41.2 (4-CH₂), 39.3 (C₃), (10.6-3-CH₃). MS (ES)⁺: 577.25 [M + H]⁺. Exact Mass calculated for C₃₆H₃₆N₂O₅: 576.26242; found: 576.26457.

Functional assays by calcium microfluorimetry. Human embryonic kidney cell line (HEK) stably transfected with rTRPM8 or h-TRPV1 were used as previously described. Briefly, cells were seeded in 96-well plates at a cell density of 30,000 cells 2 days before treatment. Buffer used was HBBS (in mM): 138 NaCl, 5.33 KCl, 1.26 CaCl₂, 0.5 MgCl₂·6H₂O, 0.4 MgSO₄·7H₂O, 4 NaHCO₃, 0.44 KH₂PO₄, 0.3 Na₂HPO₄, pH 7.4. The day out 1–3 days after cells seeded. Membrane currents and voltages were recorded by patch clamp using the whole-cell configuration. For whole-cell recordings of HEK-rTRPM8 cells, pipette solution contained (in mM) 150 NaCl, 6 CsCl, 1.5 CaCl₂, 1 MgCl₂, 10 d-glucose and 10 HEPES, adjusted to pH 7.4 with NaOH. Data were normalized response and Y = log [µM].

Data analysis. The degree of inhibition of TRPM8 channel gating was calculated by comparison to calcium increase signal elicited by test substances and menthol at 100 µM. Decrease of menthol signal was expressed as percentage of inhibition (%). All data are expressed as mean ± standard deviation (SD). Each condition was increase signal elicited by test substances and menthol at 100 µM. Decrease of menthol signal was expressed as percentage of inhibition (%). All data are expressed as mean ± standard deviation (SD). Each condition was analyzed by a two-tailed unpaired Student’s t-test. Data was considered statistically significant at p < 0.05.

Functional assays by Patch-clamp electrophysiology. Electrophysiological recording was carried out 1–3 days after cells seeded. Membrane currents and voltages were recorded by patch clamp using the whole-cell configuration. For whole-cell recordings of HEK-rTRPM8 cells, pipette solution contained (in mM) 150 NaCl, 5 EGTA, 3 MgCl₂ and 10 HEPES, adjusted to pH 7.2 with NaOH, and bath solution contained (in mM) 150 NaCl, 6 CsCl, 1.5 CaCl₂, 1 MgCl₂, 10 d-glucose and 10 HEPES, adjusted to pH 7.4 with NaOH. Data were sampled at 10 kHz (EPC10 amplifier with PatchMaster 2.53 software, HEKA Electronics, Lambrecht, Germany) and low-pass filtered at 3 kHz for analysis (PatchMaster 2.53 and GraphPad Prism 5, Graphpad Software, USA). The series resistance was <10 MΩ and to minimize voltage errors was compensated to 60–80%. All measurements were performed at 24–25 °C. The TRPM8 response was quantified as the ratio P2/P1, being P2 the second increase signal elicited by test substances and menthol at 100 µM. Decrease of menthol signal was expressed as percentage of inhibition (%). All data are expressed as mean ± standard deviation (SD). Each condition was analyzed by a two-tailed unpaired Student’s t-test. Data was considered statistically significant at p < 0.05.

Docking Studies. The molecular model for rat TRPM8 was obtained using the structure of the TRPM8 from Ficedula albicollis determined by cryo-electron microscopy at 4.01 resolution. The model was used to study the interactions of the molecules with the receptor. The program then performed a simulated annealing optimization of the complexes, which moved the structure to a nearby stable energy minimum, by using the implemented Assisted Model Building with Energy Refinement (AMBER03) force field. The Yasara pH command was set to 7.0, to ensure that molecules preserved their pH dependency of bond orders and protonation patterns. The best binding energy complex in each cluster was stored, analyzed, and used to select the best orientation of the interacting partners.
Antitumor activity. Cell lines. Human tumor cell lines used in this study were purchased from the ATCC. A-549 (CCL-185), lung carcinoma; HT-29 (HTB-38), colorectal adenocarcinoma; MDA-MB-231 (HTB-26), breast adenocarcinoma; PSN1, pancreas adenocarcinoma.

Cell culture. All cell lines were maintained in DMEM (Dulbecco's Modified Eagle's Medium) culture medium supplemented with 10% FBS (Fetal Bovine Serum), 100 Units/mL penicillin/streptomycin at 37 °C, 5% CO₂, and 95% humidity.

Cytotoxicity Assay. Triplicate cultures were incubated for 72 h in the presence or absence of test compounds in dose–response curves (10 concentrations, typically ranging from 10 to 0.0026 µg/mL). A colorimetric assay using sulforhodamine B (SRB) was adapted for quantitative measurement of cell growth and cytotoxicity32. A more detailed information on this assay is provided in the supplementary information. GI₅₀ is the compound concentration that produces 50% inhibition on cell growth as compared to control cells.

In vivo anti-allodynic effects. Male C57-mice (~30 g) (Harlam, Holland) were used for the study. All experiments were approved by the Institutional Animal and Ethical Committee of the Universidad Miguel Hernández where the experiments were conducted and they were in accordance with the guidelines of the Economic European Community and the Committee for Research and Ethical Issues of the International Association for the Study of Pain. All parts of the study concerning animal care were performed under the control of veterinarians.

As previously described34, oxaliplatin (Tocris) was dissolved in water with gentle warming and was subcutaneously (s.c.) injected on days 1, 3 and 5 at a 6 mg/kg dose. The day 7 after administration, experiments were performed. Together with Oxaliplatin injection, saline and a 5% Mannitol solution were intraperitoneally injected to prevent kidney damage and dehydration. The compound 24a stock was prepared in DMSO (Sigma-Aldrich) and diluted in saline for injections. Compound at different doses (1 to 3 µg) was injected into the plantar surface (25 µL) of the right hind paw of mice. Cold chemical thermal sensitivity was assessed using acetone drop method. Mice were placed in a metal mesh cage and allowed to habituate for approximately 30 min in order to acclimatize them. Freshly dispersed acetone drop (10µL) was applied gently on to the mid plantar surface of the hind paw. Cold chemical sensitive reaction with respect to paw licking was recorded as a positive response (nociceptive pain response). The responses were measured for 20-s with a digital stopwatch. For each measurement, the paw was sampled twice and the mean was calculated. The interval between each application of acetone was approximately 5 min.

Data availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
Idea: R.G.M.; Synthesis and characterization: M.A.B., C.M.E.; In vitro activity at TRPs: R.T.M., A.M., A.F.C.; Antitumor activity: C.C., A.F.; In vivo activity: S.G.R.; Modeling: R.T.M., G.F.B.; Supervision: A.M.R., A.F.M., A.F.C., R.G.M. This article has been written though the contribution of all authors, which have approved the final version.

Competing interests
The authors declare no competing interests.

Additional information
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