ORIGINAL RESEARCH PAPER

Natural *α,β*-unsaturated lactones inhibit neuropeptide-induced mast cell activation in an in vitro model of neurogenic inflammation

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Abstract

Introduction Mast cells are involved in not only inducing, but also maintaining neurogenic inflammation and neuropathic pain. In previous work, we have demonstrated that dehydroleucodine, xanthatin and 3-benzyloxymethyl-5*H*-furan-2-one inhibit rat peritoneal and human LAD2 mast cell degranulation induced by compound 48/80 and calcium ionophore A23187. However, the effect of these molecules on neuropeptide-induced mast cell activation has not been studied so far.

Objective The aim of this study was to determine whether dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one inhibit neuropeptide-induced mast cell activation.

Methods This work is based on in vitro simulation of a neurogenic inflammation scenario involving neuropeptides and mast cells, to subsequently analyze potential therapeutic strategies for neuropathic pain.

Results Neuromedin-N did not stimulate mast cell serotonin release but substance *P* and neurotensin did induce serotonin release from peritoneal mast cells in a dose-dependent manner. Mast cell serotonin release induced by substance *P* and neurotensin was inhibited by dehydroleucodine and xanthatin, but not by 3-benzyloxymethyl-5*H*-furan-2-one. The inhibitory potency of dehydroleucodine and xanthatin was higher than that obtained with the reference compounds, ketotifen and sodium chromoglycate, when mast cells were preincubated with dehydroleucodine before substance *P* incubation, and with dehydroleucodine or xanthatin before neurotensin incubation.

Conclusions These results are the first strong evidence supporting the hypothesis that dehydroleucodine and xanthatin inhibit substance *P*- and neurotensin-induced serotonin release from rat peritoneal mast cells. Our findings suggest, additionally, that these *α,β*-unsaturated lactones could be of value in future pharmacological research related to inappropriate mast cell activation conditions such as neurogenic inflammation and neuropathic pain.

Keywords Mast cells · Substance *P* · Neurotensin · Neuromedin · Dehydroleucodine · Xanthatin

Introduction

Mast cells are immunocytes of hematopoietic origin with secretory functions. Inflammatory mediators such as histamine, heparin, serotonin, prostaglandins, cytokines are secreted from mast cells when they are activated by either immunological (e.g., IgE) or non-immunological stimuli (e.g., neuropeptides, calcium ionophore, compound 48/80) [3, 4, 21, 24, 25, 29].

For years, mast cells have been mainly studied for their involvement in anaphylactic and allergic responses [15]. However, at present, these cells are recognized for their role in several other pathological and physiological processes. Mast cells contribute to the pathogenesis of inflammatory diseases such as rheumatoid arthritis, scleroderma, interstitial cystitis, multiple sclerosis, irritable bowel disease, and neuropathic pain, as well as in processes such as wound healing, angiogenesis, and the development of tumors [9, 16, 25, 26, 31, 33, 39].

Mast cells are the first responder cells of the immune system [23], and some research has put these cells at the center stage of immunology [22]. There is growing evidence that mast cells are the leader cells of inflammation and the

prototypical neuroimmune cell [12, 37]. In physiological levels, inflammation is a critical biological process, as well as a self-limiting and protective condition. However, in high doses, this response can cause an acute inflammation reaction that, in turn, can eventually produce chronic inflammation and lead to a variety of diseases such as neurogenic inflammation, the substrate of neuropathic pain [10, 37]. It is well known that the immune system interacts with the sensory nervous system contributing to diverse pain states, and there is increasing information that inflammation is a cause of nociceptive pain. However, nociceptive pain differs from neuropathic pain, which arises as a consequence of a disease or lesion of the somatosensory system. In some instances, this lesion may be attributable to an aberrant immune response resulting in excessive neuroinflammation of the peripheral or central nervous system [10].

In this regard, mast cells are particularly important because they are frequently found close to the vasculature, and sensory nerve endings or nociceptive neurons, and and thus can participate in juxtacrine signaling in neuroimmune synapses. Therefore, mast cells and their inflammatory mediators are involved in not only inducting but also maintaining neurogenic inflammation and neuropathic pain caused by various stimuli [8].

Pain information begins at the nerve endings called nociceptors, which form a functional pain unit with the nearby tissue capillaries. Following injury or inflammatory stimuli, preformed preactivated and granule-stored neuroactive inflammatory such as histamine and serotonin, among others, are released by degranulation from mast cells and stimulate nociceptive afferents [16]. Growth factors and many newly synthesized neuroactive and neuroinflammatory mediators are also released from mast cells. In response to this activation, nociceptive fibers, themselves, release neuromodulators such as substance P, neurotensin, and neuromedin, which, in turn, can stimulate the activation of mast cells. Subsequently, mast cells amplify vasodilation and sensitize nociceptors. This positive feedback loop causes neurogenic inflammation. Mast cell stabilizers such as cromolyn or ketotifen can inhibit nociception and neurogenic inflammation [12, 16, 18, 19, 27, 36, 40]. Mast cells also contribute to the recruitment of other immune cells such as neutrophils, macrophages, and T cells, which release pro-nociceptive mediators and reinforce the maintenance of inflammatory reactions. As a consequence, inflammation can affect not only injured zones but also adjacent territories, creating secondary, wide-spread hyperalgesia, which is an excessive pain response [16].

Neuropathic pain is associated with significant medical care costs and low labor productivity [34]. Current therapeutic approaches are mainly aimed at modulating mast cell numbers, inhibiting mast cells, preventing mast cell activation and modulating mast cell signal transduction, and

protection from the effects of mast cell mediators.[31]. Thus, targeting mast cells provides a potentially treatable target for disorders of the central nervous system as pain [18]. In this context, analyzing how mast cells interact with molecules involved in neurogenic inflammation could be a promising field for the treatment of neuropathic pain.

In previous work, we have demonstrated that three α,β-unsaturated lactones called: (1) dehydroleucodine (a sesquiterpene lactone isolated from *Artemisia douglasiana* Besser, popularly known as "matico"), (2) xanthatin (a xanthanolide sesquiterpene isolated from *Xanthium cavanillesii* Schouw, popularly known as "abrojo grande") and (3) 3-benzyloxymethyl-5*H*-furan-2-one (a semisynthetic butenolide obtained in our laboratory), inhibit rat peritoneal and human LAD2 mast cell degranulation induced by compound 48/80 and calcium ionophore A23187 [28, 38]. However, the effect of these molecules on neuropeptide-induced mast cell activation has not been studied so far.

This work is based on in vitro simulation of a neurogenic inflammation scenario involving neuropeptides and mast cells to subsequently analyze potential therapeutic strategies for neuropathic pain. The aim of this study was to determine whether dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one inhibit neuropeptide-induced mast cell activation.

Materials and methods

Chemicals and reagents

Substance P, neurotensin, neuromedin-N, ketotifen, sodium chromoglycate, bovine serum albumin (fraction V), dimethyl sulfoxide (DMSO), serotonin standard for high-performance liquid chromatography (HPLC), toluidine blue, trypan blue, and formaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Substance P and neurotensin were dissolved in physiological saline (stock solutions: 50 mg/ml and 10 mg/ml, respectively) and stored at – 80 °C until required. The stock solutions were then diluted with physiological saline to the desired final concentration. Dehydroleucodine, xanthatin and 3-benzyloxymethyl-5*H*-furan-2-one were dissolved in DMSO (stock solutions: 100 mg/ml) and stored at – 80 °C until required. The stock solutions were then diluted with physiological saline to the desired final concentration. The final concentration of DMSO in reagent media was less or equal than 1%. Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). HPLC solvents were purchased from Mallinckrodt (St. Louis, MO, USA). All other substances were supplied by Merck (Darmstadt, Germany). All chemicals used in these studies were of the highest grade available.

Ethics statement

All animal experiments were evaluated and approved by the CICUAL (Institutional Committee for Care and Use of Laboratory Animals), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo. Regulations of this Committee are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH, USA) to comply with established international regulations and guidelines.

Animals

Wistar adult rats ($n = 70$) weighing approximately 300–500 g, infection-free, and maintained under a 12-h dark/light cycle in a temperature-controlled room (24–25 °C) with free access to drinking water and laboratory food, were used for this work.

Isolation and purification of dehydroleucodine

Artemisia douglasiana Besser was collected in the mountains of the province of San Luis, Argentina, in March 2015, and was identified by Prof. Luis A. Del Vitto. A voucher specimen was deposited in the Herbarium of the Universidad Nacional de San Luis (UNSL no.55). Dehydroleucodine was isolated as previously described [14]. Briefly, the air-dried material (1 kg) was soaked in chloroform, at room temperature. The extracts were vacuum-dried and redissolved in 95% ethanol. After the addition of a 4% aqueous lead tetraacetate solution, the cloudy aqueous solution was filtered through a celite pad, and the filtrate was concentrated under vacuum. The mixture was extracted three times with chloroform, and the solution was concentrated under vacuum. After evaporation of the solvent, the resulting residue was fractionated by repeated flash chromatography and column chromatography. The chromatographic purification of the chloroform extract yielded dehydroleucodine (8 g, 100% purity), which was identified by [1]H- and ^{13}C-nuclear magnetic resonance, mass spectrometry, infrared spectra, and melting point analysis.

Isolation and purification of xanthatin

Xanthium cavanillesii Schouw was collected in El Volcán, Dpto. La Capital, San Luis, Argentina, in March 2015, and was identified by Prof. Luis A. Del Vitto. A herbarium sample is available from the Herbario of the Universidad Nacional de San Luis (voucher 8985-UNSL). Xanthatin was isolated as previously described [11]. Briefly, the air-dried aerial parts of this plant (4.8 kg) were extracted with acetone (three times) at room temperature for 5 days. The extract was concentrated under vacuum, and the resultant dark brown syrup (560 g) was dissolved in a mixture of methanol:water (9:1), filtered, and extracted with *n*-hexane to remove pigment and fatty materials. The hydroalcoholic solution was diluted with water (7:3) and then extracted with chloroform. After solvents were evaporated, the resulting residue was fractionated by repeated flash chromatography and column chromatography. This purification protocol yielded xanthatin (3.8 g, 100% purity), which was identified by [1]H- and ^{13}C-nuclear magnetic resonance, mass spectrometry, infrared spectra, and melting point analysis.

Preparation of 3-benzyloxymethyl-5H-furan-2-one

3-benzyloxymethyl-5*H*-furan-2-one was prepared as previously described by Ceñal et al. [7]. Briefly, the general procedure to obtain butenolides from furan was applied to 3-benzyloxymethyl-furan on a 1200 mg (6.37 mmol) scale, yielding the butenolide 3-benzyloxymethyl-5*H*-furan-2-one (1050 mg, 5.14 mmol, 80.6%). 3-benzyloxymethyl-5*H*-furan-2-one (100% purity, flash chromatography) was identified by [1]H- and ^{13}C-nuclear magnetic resonance, mass spectrometry, infrared spectra, and melting point analysis.

Isolation and purification of mast cells

Mast cells were isolated by peritoneal lavage as previously described [2] with some modifications. In brief, rats were euthanized by CO_2 inhalation. Then, 30 ml of a solution containing 6.7 mM Na_2HPO_4, 6.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.8 mM $CaCl_2$, 0.5 g/l albumin, and 1 g/l glucose, adjusted to pH 7.2 was injected into the peritoneal cavity through a small incision in the abdominal wall. The abdomen was gently massaged for about 3 min. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was extracted with a Pasteur pipette. Peritoneal mast cells were then purified by centrifugation through a discontinuous gradient of Percoll [17]. Harvesting of the mast cells posed no problem since these cells gathered in a layer at the bottom of the tube, whereas other cells formed a rather compact layer on top of the gradient and could easily be removed by aspiration. Cells were stained metachromatically with toluidine blue (0.1% w/v, pH 1.0) and quantified using a Neubauer hemocytometer under a Nikon microscope (magnification 400×). Crude peritoneal cell suspensions contained 3% mast cells, and the purity of the mast cells after gradient centrifugation was more than 90%. Purified mast cells were washed, resuspended in balanced salt solution containing 6.7 mM Na_2HPO_4, 6.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.8 mM $CaCl_2$, 0.5 g/l albumin, and 1 g/l glucose, adjusted to pH 7.2 (cell density of 2×10^6/ml), and maintained for a maximum of 30 min at 4 °C. Mast cells were considered viable upon their

ability to exclude trypan blue and by measuring serotonin in the supernatant. The trypan blue exclusion test indicated a viability of over 95%. Nonspecific, spontaneous serotonin release was always less than 6%.

General protocol

Purified peritoneal mast cells (cell density of 1×10^7/ml) were equilibrated at 37 °C for 10 min.

The first set of experiments was performed to determine the concentration of each neuropeptide that would induce maximum mast cell degranulation without producing cytotoxic effects. First, 100 μl aliquots of the equilibrated cells were preincubated with a balanced salt solution in polypropylene tubes for 10 min at 37 °C. Next, incubation with increasing concentrations of substance P, neurotensin or neuromedin-N for additional 10 min at 37 °C (10, 50, and 100 μM of each neuropeptide) was performed.

The second set of experimentations tested the effect of dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5H-furan-2-one on mast cell degranulation induced by the neuropeptides. First, 100 μl aliquots of the equilibrated cells were preincubated with the test lactones (dehydroleucodine, xanthatin, or 3-benzyloxymethyl-5H-furan-2-one) in polypropylene tubes for 10 min at 37 °C and then incubated with substance P or neurotensin (final concentration of each of the neuropeptides: 100 μg/ml) for 10 min at 37 °C. Preincubated cells were not activated with neuromedin-N, because this neuropeptide did not stimulate mast cells in this experimental model. Negative (no stimulation with the neuropeptides) and positive (stimulation with 100 μg/ml of the neuropeptides) controls were included in all experiments. Dose–response dependence studies were conducted using concentrations of dehydroleucodine, xanthatin, or 3-benzyloxymethyl-5H-furan-2-one of 4, 12, 20, 40, 80, and 160 μM. Different lactone combinations were also tested.

The final volume of incubation in each tube was 500 μl. The mean total number of mast cells during incubations was 2×10^6/ml per tube. The secretion was stopped by cooling the tubes in an ice-cold water bath. Cells and supernatants were separated by centrifugation (180 g, 5 min, 4 °C). The supernatants were used for the determination of serotonin content by HPLC, which was taken as a measure of serotonin release. Serotonin release was used as a valuable biochemical marker of mast cell activation [41]. The cell pellets were vortexed for 5 s and then sonicated for 1 min, in 0.1 N HCl, to liberate the residual serotonin, which was quantified by HPLC and taken as a measure of remaining serotonin. Ketotifen and sodium chromoglycate, two reference mast cell stabilizers, were used to evaluate the potency of

the compounds dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5H-furan-2-one. Other samples of cell pellets were used for cell viability studies by the trypan blue dye exclusion test or fixed for light microscopy. Cell viability studies were carried out to ensure that changes in serotonin release were not due to cell death. The percentage of serotonin release in each tube was calculated using the formula: serotonin release (%) $= 100S/(S + C)$, where S is serotonin content of the supernatant, and C is serotonin content of the residual cells. The spontaneous serotonin release after incubations was always less than 10%.

Finally, a set of incubations were performed to evaluate, by thin-layer chromatography, the stability of the α,β-unsaturated lactones along the incubation periods, and to exclude the possibility that the lactones could interact with the neuropeptides during the incubations. All the experiments were repeated at least five times in duplicate.

High-performance liquid chromatography

Serotonin levels were measured by high-performance liquid chromatography (HPLC) with electrochemical detection, as previously described [1], with modifications as follows. A 10-μl aliquot of the solution was filtered through a 0.22 μm Millipore filter and injected directly into the HPLC apparatus. The HPLC system consisted of a pump (Isco 2350, USA) to deliver the mobile phase, linked to a sample injector (Valco C6W, USA) with a 10 μl sampling loop, and a separation column (25 cm × 4.6 mm i.d., 5 μm particle size, Macherey–Nagel Nucleosil C18, USA). The electrochemical detector (Shimadzu L-ECD-6A, Japan) contained a glassy-carbon working electrode whose potential was maintained at + 0.6 V versus a silver–silver chloride reference electrode. The mobile phase consisted of 0.02 mol/L phosphate buffer (pH 3.0) containing 0.3 mM EDTA and 2% (v/v) acetonitrile. The flow rate was 1 ml/min. The mobile phase was filtered through a 0.45 μm Millipore filter under vacuum and degassed before use. All chromatograms were recorded and analyzed using a computer program (SRI PEAKSIMPLE II, USA). In the selected experimental conditions, serotonin exhibited a well-defined chromatographic peak with a retention time of 10.1 ± 0.05 min. Serotonin release was expressed as the percentage of serotonin release.

Light microscopy

Mast cells were fixed in 2% formaldehyde. After 2 h in the fixative, cell suspensions (2×10^5/ml) were stained with toluidine blue (0.1% w/v, pH 3.0), placed between slides and cover slides, and examined under a Nikon 80i microscope.

Thin-layer chromatography

Incubation solutions were checked, before and after incubations, by thin-layer chromatography to evaluate the lactone stability and reactivity. The incubation solutions were extracted with ethyl ether (three times) at room temperature. The extracts were concentrated under vacuum, and the residues were dissolved in ethyl acetate. Dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one standards were also dissolved in ethyl acetate. Standard solutions and incubation solution extracts were applied to thin-layer chromatography (TLC) plates (Silica gel 60 F_{254}, Merck, Darmstadt, Germany), developed to the top of the TLC plates with ethyl acetate/*n*-hexane (40:60), and visualized with potassium permanganate staining. Ratios of front (Rf) values were calculated for individual bands by the following equation: Rf = (distance the spot moves)/(distance to the solvent front). Rf values from incubation solution extracts were compared to those of standard solutions.

Statistical analysis

Results from biochemical studies are presented as means ± S.E.M. Differences between groups were determined using analysis of variance followed by the Tukey–Kramer multiple comparisons test. $P < 0.05$ was considered statistically significant.

Results

Effect of increasing concentrations of substance P, neurotensin and neuromedin-N on mast cell serotonin release

First, we needed to ensure that substance *P*, neurotensin, and neuromedin-N induced serotonin release from mast cells in our experimental model. It was also necessary to determine the dose of each neuropeptide that would stimulate the highest release of serotonin without causing cell death. Peritoneal mast cells were incubated with increasing concentrations of the neuropeptides to respond to these issues. Serotonin release and cell viability were analyzed after the incubations.

Figure 1 shows the effect of varying concentrations of substance *P*, neurotensin, and neuromedin-N on serotonin release from rat peritoneal mast cells (Fig. 1a, c, e, respectively) and mast cell viability (Fig. 1b, d, f, respectively). The neuropeptides substance *P* and neurotensin induced a significant serotonin release from peritoneal mast cells in a dose-dependent manner without affecting cell viability. The dose of both neuropeptides that stimulated the highest release of serotonin without causing cell death was 100 µM. In contrast,

neuromedin-N did not stimulate the release of serotonin from mast cells at any of the concentrations used. Higher doses from those used in the experiments were accompanied by a significant decrease in cell viability (data not shown).
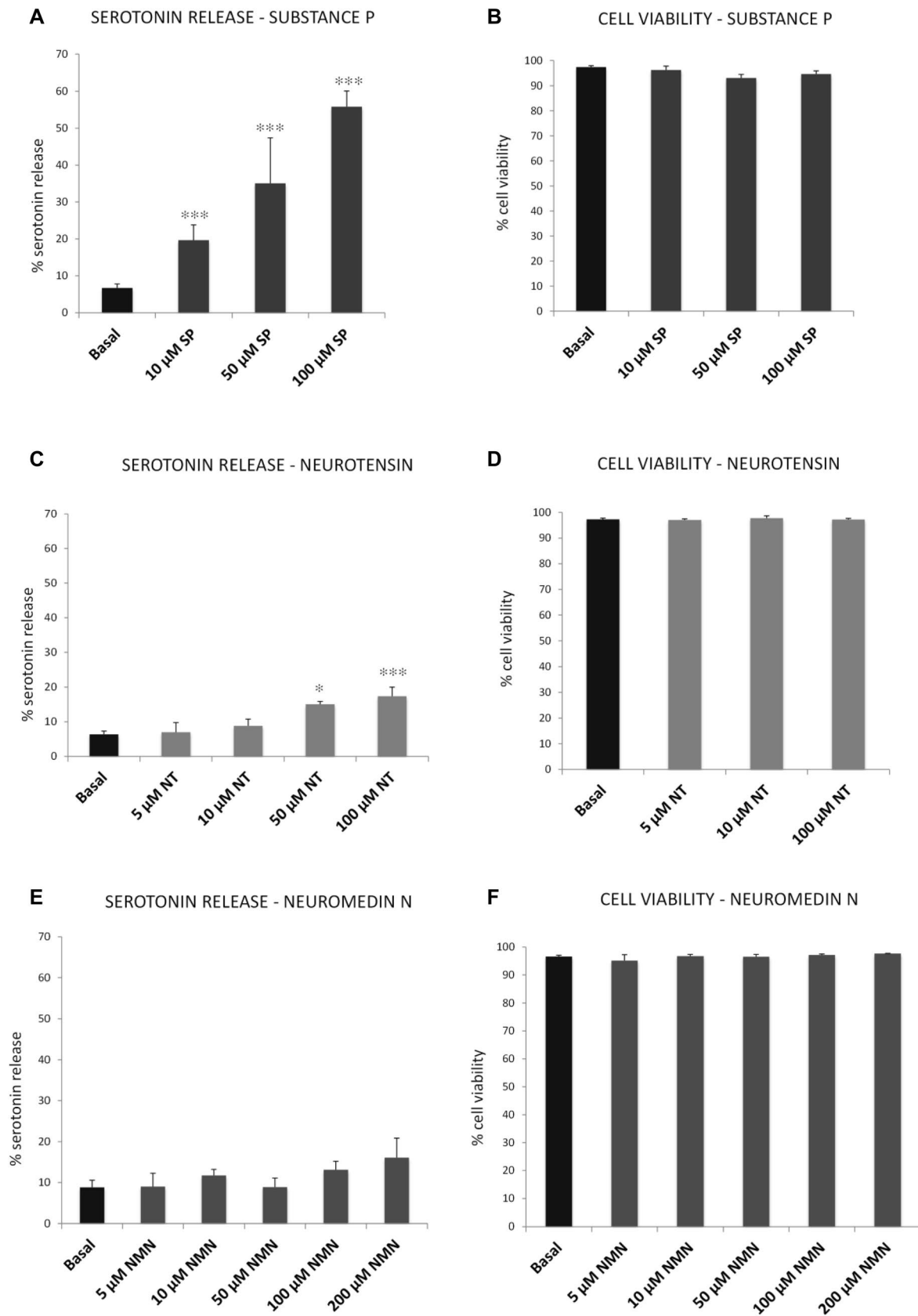
Effect of dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one on mast cell serotonin release induced by substance *P* or neurotensin

Here, we examined whether dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one inhibit mast cell serotonin release induced by 100 µM substance P or by 100 µM neurotensin. The results show that dehydroleucodine and xanthatin inhibit serotonin release induced by substance *P* and by neurotensin (Figs. 2, 3). This inhibitory action was not accompanied by changes in cell viability, except for dehydroleucodine concentrations higher than 160 µM. However, 3-benzyloxymethyl-5*H*-furan-2-one did not inhibit serotonin release from mast cells at any of the concentrations used (Figs. 2, 3).

Then, a comparative efficacy study was performed with ketotifen and sodium chromoglycate (chromolyn), two reference mast cell stabilizers. Cromolyn is especially interesting, because it is a well-described mast cell stabilizer, and its structure is also based on *α,β*-unsaturated lactone group, as well as dehydroleucodine, xanthatin and 3-benzyloxymethyl-5*H*-furan-2-one. The inhibitory effects were higher than those obtained with the reference compounds ketotifen and sodium chromoglycate when mast cells were preincubated with dehydroleucodine before substance P stimulation, and with dehydroleucodine or xanthatin before neurotensin challenge. The values of IC50 for each compound are shown in Table 1. The order of potency in this experimental model was dehydroleucodine > ketotifen > sodium chromoglycate > xanthatin > 3-benzyloxymethyl-5*H*-furan-2-one (before substance *P* stimulation) and xanthatin > dehydroleucodine > ketotifen > sodium chromoglycate > 3-benzyloxymethyl-5*H*-furan-2-one (before neurotensin challenge).

We also examined whether different combinations of the lactones could overcome the inhibitory effects observed with dehydroleucodine + substance *P* and with dehydroleucodine/xanthahtin + neurotensin. None of the tested combinations exceeded the previously described potent inhibitory effects (Fig. 4).

Dehydroleucodine, xanthatin, or 3-benzyloxymethyl-5*H*-furan-2-one applied alone did not affect the mediator release, and no significant differences were detected in the total cell serotonin content (released + remnant) between groups. Serotonin release induced by 1% DMSO was

A SEROTONIN RELEASE - SUBSTANCE P

B CELL VIABILITY - SUBSTANCE P

C SEROTONIN RELEASE - NEUROTENSIN

D CELL VIABILITY - NEUROTENSIN

E SEROTONIN RELEASE - NEUROMEDIN N

F CELL VIABILITY - NEUROMEDIN N

◀**Fig. 1** Effect of varying concentrations of substance *P*, neurotensin, and neuromedin-N on serotonin release from rat peritoneal mast cells. Results are expressed as the percentage of serotonin release (**a**, **c**, and **e**) and as the percentage of cell viability (**b**, **d**, and **f**). Values are presented as means ± S.E.M. **P < 0.05* and ****P < 0.001* versus basal. *SP* substance *P*, *NT* neurotensin, *NMN* neuromedin-N

similar to that from cells incubated with buffer alone (data not shown).

Regarding morphological studies, peritoneal mast cells were easily identified by the presence of a cytoplasm dominated by distinctive secretory granules which stain metachromatically (Fig. 5). Mast cells from the basal group are dominated by tightly packed secretory granules. Cell surface disruption and degranulation may be seen in mast cells stimulated with the neuropeptides. The morphology of the cells treated with dehydroleucodine or xanthatin shows a lower degree of degranulation than that of secretagogue samples. Mast cells treated with 3-benzyloxymethyl-5*H*-furan-2-one before the neuropeptides show the same appearance as that of the neuropeptides group.

Thin-layer chromatography

The Rf values were 0.49, 0.46, and 0.51 for dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one, respectively. Neither changes in Rf values for each lactone nor degradation bands were observed in the plates after incubations.

Discussion

The first findings of the present study show that the neuropeptides substance *P* and neurotensin induce a significant serotonin release from rat peritoneal mast cells in a dose-dependent manner. The stimulatory effect of substance *P* is higher than that exhibited by neurotensin at the same concentrations. However, the neuropeptide neuromedin-N does not stimulate the release of serotonin from mast cells at any of the concentrations used.

Substance *P* is an undecapeptide that derives from *α*, *β*, and *γ* pre-protachykinin gene transcripts. The potency of substance *P* in degranulating mast cells had already been found decades ago. The effects of substance *P* on mast cells are mediated either through the neurokinin-1 receptor (NK-1R) or Mas-related G protein-coupled receptor (MRGPRX2 in humans; Mrgprb2 in mice and rats) [6]. It has also been reported that the ability of substance P to induce histamine

release seems to appear only at high concentrations [32]. Both NK-1R and MRGPRX2/Mrgprb2 are G-protein-coupled receptors (GPCRs). These receptors comprise a family of over 800 genes encoding proteins with 7-transmembrane spanning helical domains. GPCRs are associated with a trimeric GTP-binding protein (G protein) composed of Gα and Gβγ subunits. Upon activation, GDP dissociates from Gα, allowing GTP to bind, resulting in dissociation from Gβγ. Gα can be grouped into four classes: Gs, Gi, Gq, and $G_{12/13}$ which couple to different signaling effectors. Gs is coupled to the activation of adenylate cyclase, whereas Gi to its inhibition. Gq is coupled to the activation of PLCβ and $G_{12/13}$ couples to various effectors such as PLCε or RhoGEFs. The Gβγ subunits also regulate intracellular effectors such as PLCβ. MRGPRX2/Mrgprb2 are expressed abundantly in the neuronal system in dorsal root ganglia, but mast cells are the only other cells that express the MRGPRX2 isoform in humans or their orthologs Mrgprb2 and Mrgprb3 in mice and rats. MRGPRX2/Mrgprb2 couple to both Gi and Gq subunits [30].

Neurotensin is a tridecapeptide expressed in the brain and the gastrointestinal tract. However, it is also known for interacting with immune cells such as lymphocytes and mast cells. Mast cells express NTSR1132, a GPCR that couples to both Gi and Gq. Neurotensin binding induces mast cell degranulation, albeit the degranulating activity seems to be lower than that of substance *P* [30].

This background is congruent with our present results. We have shown, in our experimental model, that mast cell serotonin release induced by substance *P* is higher than that exhibited by neurotensin at the same concentrations. This could be explained on the basis that substance *P* functions through the activation of two receptors, while neurotensin only activates one receptor. Substance *P* stimulates the Mas-related G protein-coupled receptor (MRGPRX2/Mrgprb2) in addition to its conventional receptor, neurokinin-1 (NK-1R). Neurotensine only activates the NTSR1 receptor [30].

Neuromedin peptides are divided into four classes: (1) Bombesin comprising of Neuromedin B and Neuromedin C; (2) Kanassin comprising Neuromedin K and Neuromedin L; (3) Neurotensin comprising Neuromedin N; (4) Neuromedin U and Neuromedin S [13]. Neuromedin-N is a basic hexapeptide with four amino acids in common with neurotensin. Neuromedin N has a high affinity for the neurotensin receptors and acts as an agonist on these receptors. They are G protein-coupled receptors following the calcium signaling pathway [13]. Previous work has reported that neuromedin-N dose dependently stimulates the release of histamine from rat serosal mast cells at millimolar levels. However, these observations were not accompanied by
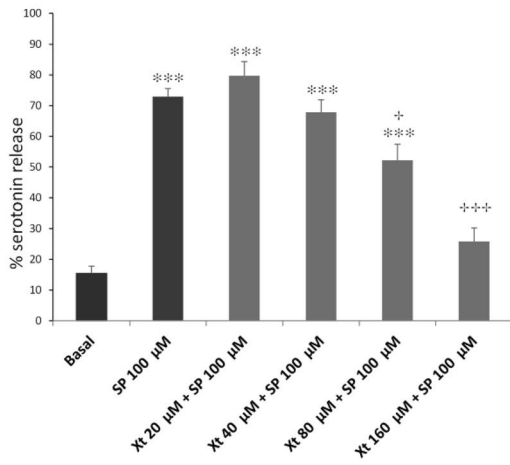
A

SEROTONIN RELEASE
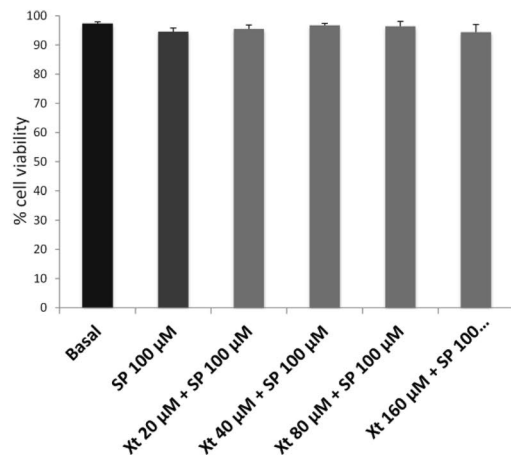DEHYDROLEUCODINE + SUBSTANCE P

B
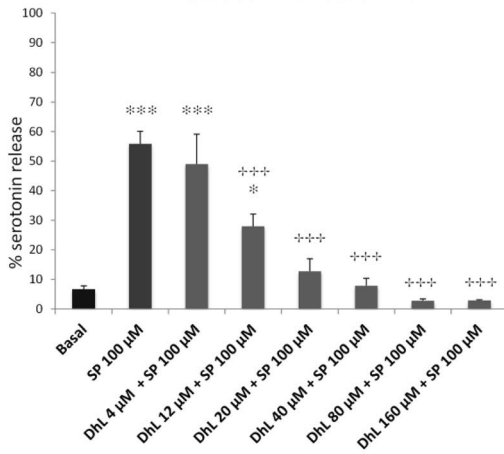
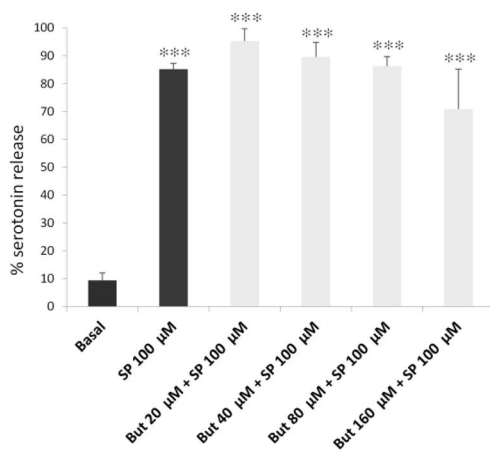CELL VIABILITY
DEHYDROLEUCODINE + SUBSTANCE P

C
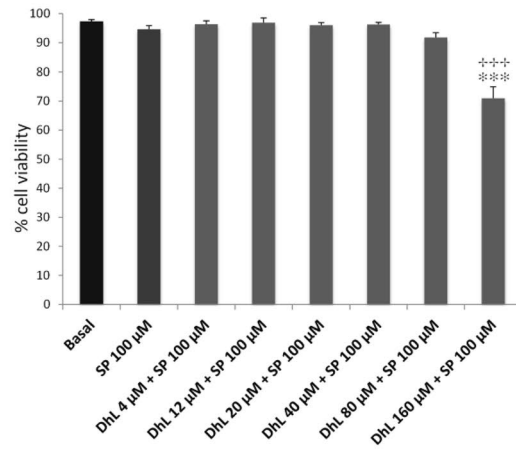
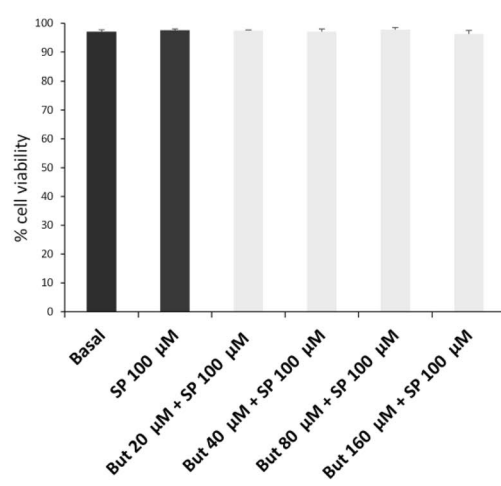SEROTONIN RELEASE
XANTHATIN + SUBSTANCE P

D

CELL VIABILITY
XANTHATIN + SUBSTANCE P

E

SEROTONIN RELEASE
3-BENZYLOXYMETHYL-5*H*-FURAN-2-ONE + SUBSTANCE P

F

CELL VIABILITY
3-BENZYLOXYMETHYL-5*H*-FURAN-2-ONE + SUBSTANCE P

◄**Fig. 2** Effect of varying concentrations of dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one on substance *P*-induced serotonin release from rat peritoneal mast cells. Results are expressed as the percentage of serotonin release (**a**, **c**, and **e**) and as the percentage of cell viability (**b**, **d**, and **f**). Values are presented as means ± S.E.M. *$P<0.05$ and ***$P<0.001$ versus basal. + + +$P<0.001$ versus 100 µM substance *P*. *SP* substance *P*, *DhL* dehydroleucodine, *Xt* xanthatin, *But* 3-benzyloxymethyl-5*H*-furan-2-one

cytotoxicity studies. The authors have also demonstrated that this effect is 10–100 times less potent than neurotensin [35]. This could be explained as follows. Because neuromedin-N shares strong homology with the C-terminal hexapeptide sequence of neurotensin, it exhibits a similar pharmacological profile to its parent peptide. The major structural difference between neurotensin and neuromedin-N lies in the fact that, unlike neurotensin, neuromedin-N is not protected at its N-terminus, thus rendering it susceptible to aminopeptidase attack. Both neurotensin and neuromedin-N are efficiently inactivated by peptidases in vitro. However, neuromedin-N is more rapidly degraded than neurotensin [20].

In our experimental model, we have demonstrated that neuromedin-N does not stimulate the release of serotonin from mast cells at non-cytotoxic doses, and that neuromedin-N concentration ranging millimolar levels induces significant cell death. Considering this first set of results, we decided to use substance *P* and neurotensin in doses of 100 µM to carry out the subsequent pharmacological tests.

We further report that incubation of peritoneal mast cells with a 100 µg/ml substance *P* or neurotensin solution increased serotonin release, and that this effect was inhibited by dehydroleucodine and xanthatin in a dose-dependent manner. However, 3-benzyloxymethyl-5*H*-furan-2-one did not inhibit serotonin release from mast cells at any of the non-cytotoxic concentrations used. The results of trypan blue exclusion tests demonstrated that the drugs were not cytotoxic at the concentrations used.

The chemistry of the lactones could explain the higher reactivity exhibited by dehydroleucodine and xanthatin. These two lactones have an exocyclic methylene group conjugated to the lactone carbonyl group, as well as a second potential reactive site as part of an unsaturated methyl-ketone substructure. Both sites constitute electrophilic centers capable of forming Michael adducts by the addition of biological nucleophiles, which are found in cells and tissues. Instead of the exocyclic reactive structure, 3-benzyloxymethyl-5*H*-furan-2-one has an endocyclic methylene group, which is less reactive than the exocyclic double bond. Moreover, 3-benzyloxymethyl-5*H*-furan-2-one lacks the other potential reactive site [7, 11, 14].

Additionally, we had revealed that the inhibitory effects exhibited by dehydroleucodine were stronger than those of the reference compounds, ketotifen, and sodium chromoglycate when mast cells were stimulated both by substance *P* and neurotensin. Instead, the inhibitory effects induced by xanthatin were stronger than those of the reference compounds, ketotifen, and sodium chromoglycate, when mast cells were stimulated by neurotensin, but not by substance *P*. This could be explained on the basis that dehydroleucodine is known to be biologically more reactive than xanthatin [28] and that the potency of substance *P* is higher than that of neurotensin [30]. However, these assertions do not fully explain the extremely high potency exhibited by xanthatin before the neurotensin challenge. Several speculations could be made about this, but it would be more responsible to carry out further studies to elucidate the mechanisms of action of these compounds at the cellular and molecular level.

Our morphological findings, at the light microscope level, reinforced the validity of our initial functional results. Microscopy studies showed that dehydroleucodine and xanthatin, but not 3-benzyloxymethyl-5*H*-furan-2-one, inhibited the release of metachromatic granules from mast cells, suggesting an interaction of the first lactones with the mast cell population and an inhibition of the degranulation induced by substance *P* or neurotensin.

The fact that mast cells treated with dehydroleucodine or xanthatin before substance *P* or neurotensin showed only minimal degranulation might indicate either: (1) changes in the ability of mast cells to adequately respond to secretagogues or: (2) inactivation of the secretagogue by degradation or chemical interaction with the lactones. The second possibility could imply structural changes in the lactones that could be detected by thin-layer chromatography. Therefore, an evaluation of the Rf values for the lactones was performed. In the first qualitative approach, our results showed no changes of Rf values after 60 min- incubation with substance *P* or neurotensin. In consequence, results from thin-layer chromatography studies led us to propose there was neither degradation nor previous binding between lactones and the secretagogues.

A particular problem of human mast cell research is the difficulty in obtaining human cell material for in vitro studies. Therefore, most in vitro mast cell experiments have been carried out either with human cell materials that might have limited functional significance, such as transformed cell lines or partially immature mast cells (e.g., LAD2 cells), or with murine primary mast cells that can be easily obtained, such as peritoneal mast cells. Murine peritoneal cells have been widely used, as a single peritoneal lavage yields large numbers of mast cells that can be easily purified [3]. Therefore, rat peritoneal mast cells, a primary mast cell, were
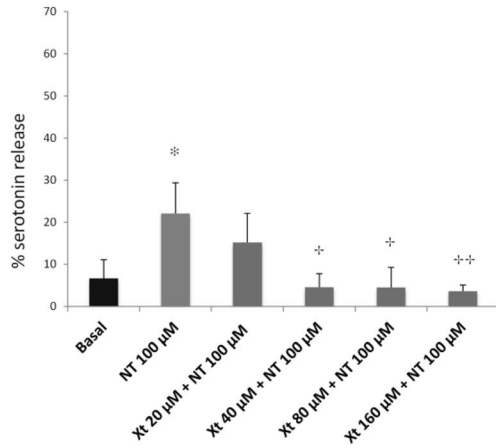
A SEROTONIN RELEASE DEHYDROLEUCODINE + NEUROTENSIN
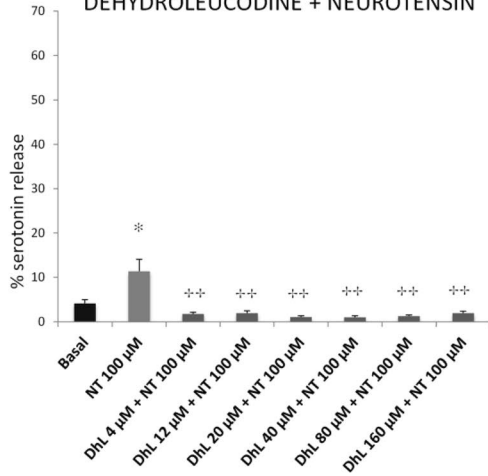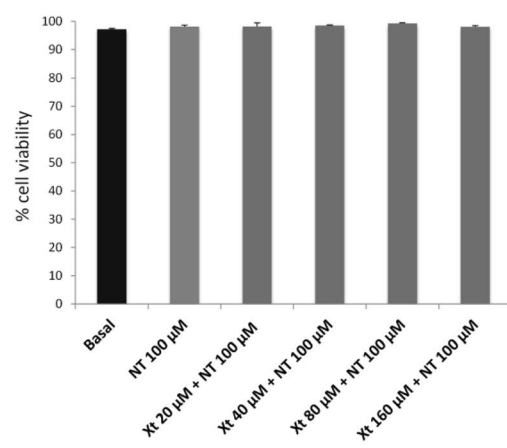
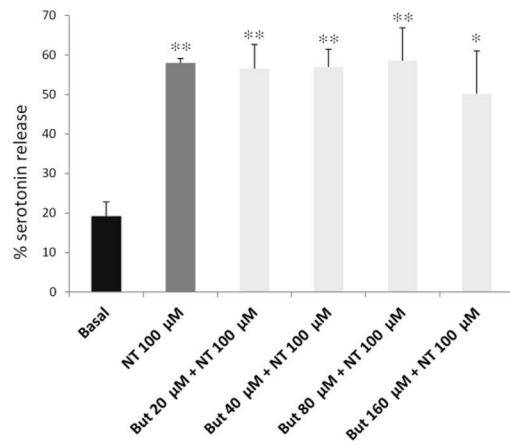**B** CELL VIABILITY DEHYDROLEUCODINE + NEUROTENSIN
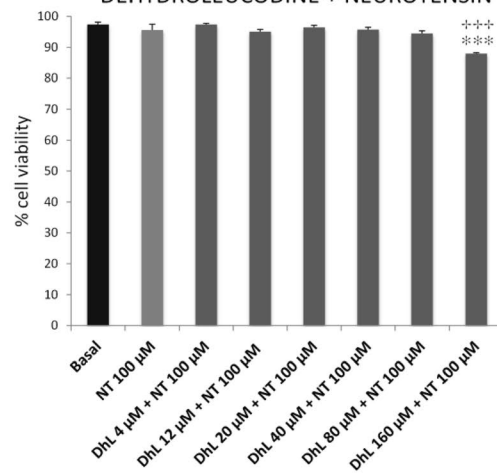
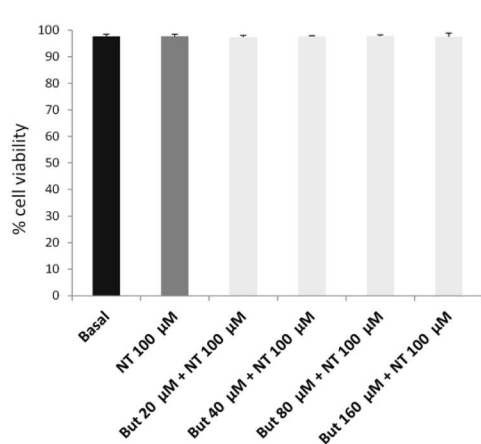**C** SEROTONIN RELEASE XANTHATIN + NEUROTENSIN

D CELL VIABILITY XANTHATIN + NEUROTENSIN

E SEROTONIN RELEASE 3-BENZYLOXYMETHYL-5*H*-FURAN-2-ONE + NEUROTENSIN

F CELL VIABILITY 3-BENZYLOXYMETHYL-5*H*-FURAN-2-ONE + NEUROTENSIN

◀**Fig. 3** Effect of varying concentrations of dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one on neurotensin-induced serotonin release from rat peritoneal mast cells. Results are expressed as the percentage of serotonin release (**a**, **c**, and **e**) and as the percentage of cell viability (**b**, **d**, and **f**). Values are presented as means ± S.E.M. *P < 0.05 and **P < 0.01 versus basal. + P < 0.05 and + + P < 0.01 versus 100 μM neurotensin. *NT* neurotensin, *DhL* dehydroleucodine, *Xt* xanthatin, *But* 3-benzyloxymethyl-5*H*-furan-2-one
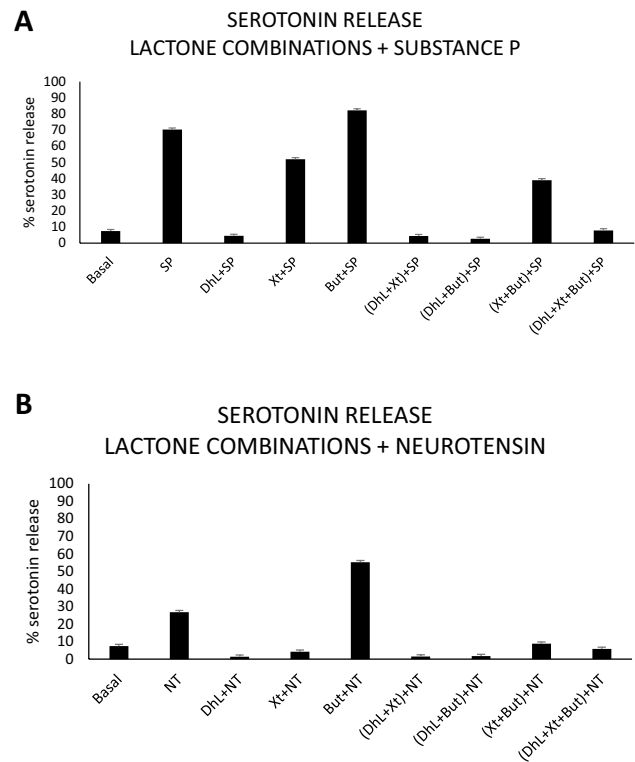
Table 1 IC50 (μM) ± SEM values for inhibitory activity of dehydroleucodine, xanthatin, 3-benzyloxymethyl-5*H*-furan-2-one ketotifen, and sodium chromoglycate on substance *P*- and neurotensin-induced mast cell activation

	Substance *P*	Neurotensin
Dehydroleucodine	7.2 ± 0.8	12.08 ± 1.1
Xanthatin	293.3 ± 30.2	0.0015 ± 0.0001
3-Benzyloxymethyl-5*H*-furan-2-one	745.662 ± 80.5	683.060 ± 70.5
Ketotifen	54 ± 5.1	40 ± 4.9
Sodium chromoglycate	97 ± 9.1	54 ± 5.1

A SEROTONIN RELEASE
LACTONE COMBINATIONS + SUBSTANCE P

B SEROTONIN RELEASE
LACTONE COMBINATIONS + NEUROTENSIN

Fig. 4 Effect of different combinations of dehydroleucodine, xanthatin, and 3-benzyloxymethyl-5*H*-furan-2-one on 100 μM substance *P*- and 100 μM neurotensin-induced (**a** and **b**, respectively) mast cell serotonin release. Combinations used were: (1) preincubation with 80 μM DhL or 80 μM Xt or 80 μM But for 10 min and incubation with 100 μM secretagogue for 10 additional min; (2) preincubation with 40 μM DhL + 40 μM Xt for 10 min and incubation with 100 μM secretagogue for 10 additional min; (3) preincubation with 40 μM DhL + 40 μM But for 10 min and incubation with 100 μM secretagogue for 10 additional min; (4) preincubation with 40 μM Xt + 40 μM But for 10 min and incubation with 100 μM secretagogue for 10 additional min; (5) preincubation with 26.7 μM DhL + 26.7 μM Xt + 26.7 μM But for 10 min and incubation with 100 μM secretagogue for 10 additional min. Values are presented as means ± S.E.M. *SP* substance *P*, *NT* neurotensin, *DhL* dehydroleucodine, *Xt* xanthatin, *But* 3-benzyloxymethyl-5*H*-furan-2-one

used for this study. Although it is not possible to establish a strict correlation between studies on human and murine cells, or between in vitro and in vivo results, the fact that dehydroleucodine and xanthatin inhibit substance *P*- and neurotensin-induced serotonin release from rat peritoneal mast cells raises the possibility that the lactones could act on human mast cells and in an intact organism. No far-reaching conclusions can be made based on these in vitro findings on rat peritoneal mast cells.

We consider that this work is relevant for several reasons:

1. the search for pharmacological strategies for pain is becoming increasingly important, because medicine has not yet provided a definitive solution to the problems of patients in pain, especially those suffering from neuropathic pain. This research may represent an exciting starting point for future studies in this sense;

2. it is essential to highlight the concept of the mast cell as a new therapeutic target in neurogenic inflammation and neuropathic pain [5]. The current trend in anesthesiology and pain is the search for drugs or mechanisms of action that block the transmission or signal of pain before it reaches the brain level and even more so before it reaches the spinal cord. Our research may offer advantages in this direction. The novel pharmacological effect

described in our work could result in future research directions related to peripheral sensitization.

3. Our study results can help valorize the compounds obtained from regional plants as well as scientifically legitimize their pharmacological effects.

In conclusion, the results reported here provide the first strong evidence supporting the hypothesis that dehydroleucodine and xanthatin inhibit substance *P*- and neurotensin-induced serotonin release from rat peritoneal mast cells. Further studies of the mechanisms of this action will lead to a complete understanding of the cellular and molecular changes that occur in mast cells after lactone treatment. Our
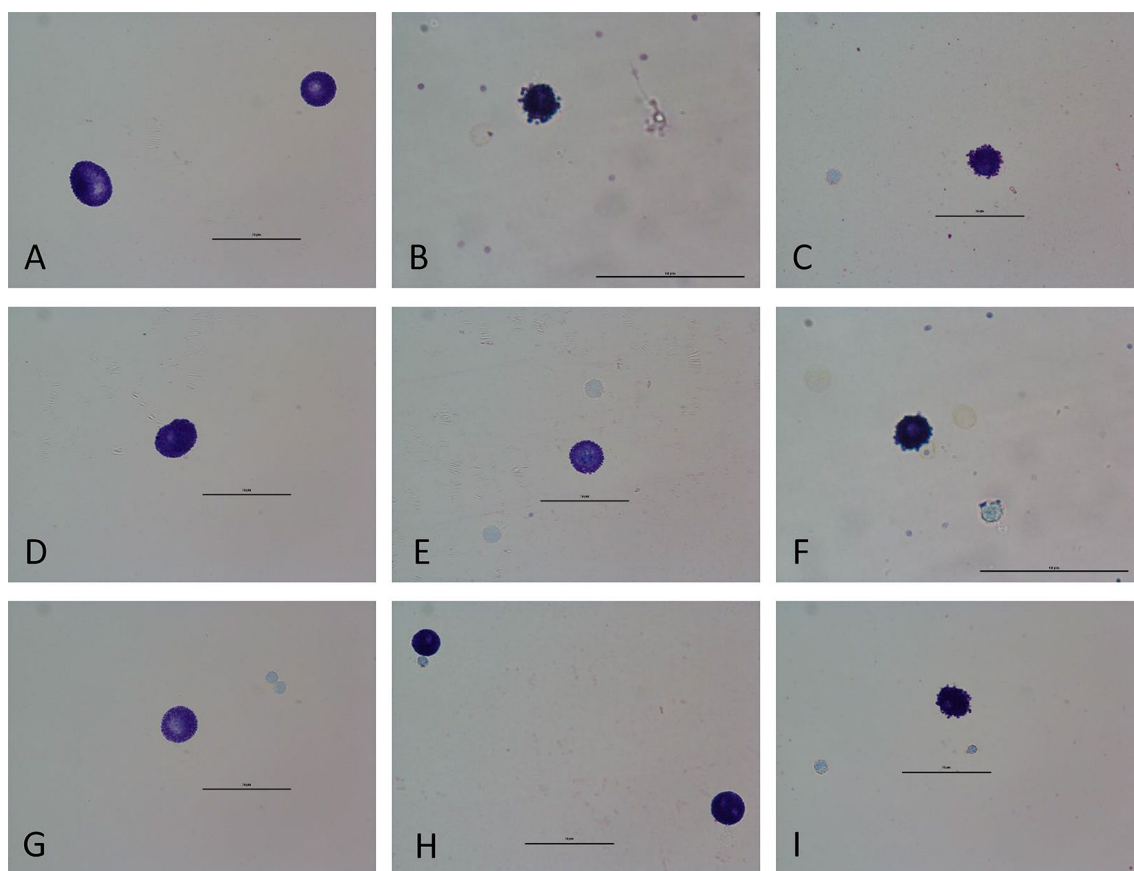
Fig. 5 Light-microscopic photographs of rat peritoneal mast cells stained with toluidine blue. Purified mast cells were preincubated with 80 μM dehydroleucodine, 80 μM xanthatin, or 80 μM 3-benzyloxymethyl-5*H*-furan-2-one for 10 min and stimulated with 100 μM substance P or 100 μM neurotensin for another 10 min at 37 °C. After incubations, cells were fixed for light microscopy. **a** basal, **b** SP, **c** NT, **d** DhL + SP, **e** Xt + SP, **f** But + SP, **g** DhL + NT, **h** Xt + NT, **i** But + NT. The figure shows a representative view of the mast cell population for each experimental group. Scale bar = 50 μm. *SP* substance *P*, *NT* neurotensin, *DhL* dehydroleucodine, *Xt* xanthatin, *But* 3-benzyloxymethyl-5*H*-furan-2-one (color figure online)

findings suggest, additionally, that these α,β-unsaturated lactones could be of value in future pharmacological research related to inappropriate mast cell activation conditions such as neurogenic inflammation and neuropathic pain.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval The Committee on the Ethics of Animal Experiments of the National University of Cuyo (Mendoza, Argentina) approved the protocol.

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