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The mechanism of action of amtolmetin guacyl, a new gastroprotective nonsteroidal anti-inflammatory drug

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Abstract

Amtolmetin guacyl (2-methoxyphenyl-1-methyl-5-*p*-methylbenzoyl-pyrrol-2-acetamido acetate) (MED15) is a new nonsteroidal anti-inflammatory drug (NSAID) with anti-inflammatory, analgesic and antipyretic properties similar to the traditional drugs, but with unexpected gastroprotective effects. In an in vivo rat model, amtolmetin guacyl administered orally demonstrates inhibition of gastric acid secretion following stimulation by various agonists, and up-regulation of gastric bicarbonate production. Pretreatment with MED15 also shows a significant reduction of indomethacin-induced gastric damage in the rat. The reason behind this behaviour appears to be bound to the presence in the MED15 molecule of a vanillic moiety known to stimulate capsaicin receptors. In fact, the antisecretive effect of MED15 is blocked by capsazepine (a specific capsaicin receptor antagonist). This effect is confirmed by the interference found with anti-histamine H_1 drugs. Owing to the connection between capsaicin and calcitonin gene-related peptide (CGRP), a possible effect of MED15 on CGRP receptors was hypothesized, considering the leading role played on gastric mucosa by the predominant sensory neuropeptide of the stomach wall, CGRP. In fact, the anti-secretive and gastroprotective effect of MED15 is abolished by CGRP-(8–37) (the specific CGRP receptor antagonist). The unmodified MED15 molecule is found throughout the gastroenteric tract for long periods of time following oral administration, as further confirmation of the mechanism of action being based on the presence of the vanillic moiety at receptor level.

Keywords: Amtolmetin guacyl; Antisecretion; Gastroprotection; Capsaicin; CGRP (calcitonin gene-related peptide)

1. Introduction

Amtolmetin guacyl (2-methoxyphenyl-1-methyl-5-*p*-methylbenzoyl-pyrrol-2-acetamido acetate), or MED15, [1] is a nonsteroidal anti-inflammatory, analgesic and antipyretic drug (NSAID), obtained through combinatorial chemistry technology: 1-methyl 5-(4-methylbenzoyl)-1*H*-pyrrole-2-acetic acid (McN-2559) was linked by an amide bond to glycine, which was in turn linked by an ester bond to a methoxyphenyl moiety (Allinger, 1977; Burkert and Allinger, 1982). Computerized conformational analysis methods were used to evaluate the spatial conformation of amtolmetin guacyl (Allinger, 1977; Burkert and Allinger, 1982). The global energetical minimum is represented by a folded structure with the external aromatic rings lying almost onto parallel planes (Allinger, 1977; Burkert and Allinger, 1982).

The spatial conformation is of the globular type, at variance with the linear conformation of McN-2559; as a result, this structural difference is probably responsible for a differential impact at receptor level, leading to different pharmacological effects (Allinger, 1977; Burkert and Allinger, 1982).

MED15, proposed for use in the wide field of rheumatic disorders, including several diseases of the connective tissue, bone, muscle, joints, nerves and blood vessels, demonstrated good gastric tolerability in pharmacological and clinical studies (Tubaro et al., 1995; Lo Giudice et al., 1998; Bianchi Porro et al., 1999).

Previous research in an in vitro model (Tubaro et al., 1995) reported that MED15 failed to reveal adverse effects on the gastric mucosa, due to marked inhibition of the

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[1] Artromed®; manufactured by Medosan Ricerca, Albano Laziale RM, Italy.

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gastric acid secretion when stimulated by various agonists. In these experiments, gross and microscopic mucosal examination raised the hypothesis of MED15-related gastric protection. Also, gastric electrical activity assessed as transmucosal potential difference was not altered by MED15, in contrast with ibuprofen and acetylsalicylic acid (Pisano et al., 1999): alteration of electrical function is in fact, known to be indicative of gastric barrier rupture (Ventura et al., 1987).

These observations are of particular importance for NSAIDs since these, although effective and beneficial drugs, are frequently associated with high incidences of gastroenteropathy, ranging from mild gastric upset to life-threatening ulceration and haemorrhage (Semble and Wu, 1987). MED15's chemical structure was implicated in the reasons underlying the characteristic pharmacological effects of the drug; in particular, the esterification of NSAIDs appears to lead to improved gastric tolerability (Samara et al., 1995).

The aim of the present study was to verify in vivo the antisecretive effects found in vitro and investigate the mechanism of action of MED15.

2. Materials and methods

2.1. Chemicals

MED15 (Alfa Chemicals Italia, Bergamo, Italy), CM-cellulose, urethane, histamine, CGRP-(8–37) and tolmetin (Sigma Aldrich, Milan, Italy), capsazepine (RBI, USA), indomethacin (I.C.F.I., Bulciago, Italy), PGE_2 kit (Amersham Italia, Milan, Italy).

2.2. Animals

Male Wistar rats 200 ± 20 g b.wt. (Harlan Nossan, Milan, Italy) quarantined for 5 days prior to the beginning of the trial and divided into groups of three animals per cage. The animals were fed standard laboratory chow in pellets (Harlan Italia, Udine, Italy) with free access to food and water under animal house conditions of 22 ± 2°C, relative humidity 55 ± 10%, 12 h light–dark cycle.

2.3. In vitro stability of MED15 in biological fluids

2.3.1. Stability in contact with plasma

Heparinized blood was collected from rats fasted overnight. MED15 was incubated at 37°C for 2, 10, 30, 60 and 180 min with plasma at a concentration of 100 μg/ml. Plasma measuring 1 ml was acidified (90 μl of HCl 1M) and mixed; the sample was then extracted with 6 ml of benzene:terbutyl alcohol (90:10 v/v) by shaking for 20 min on an automatic shaker. After centrifugation at 3000 rpm for 15 min, the organic layer was separated and dried at 40°C under a gentle stream of N_2. The residue was reconstituted with 1 ml of acetonitrile, vortex mixed, sonicated and analyzed by high performance liquid chromatography (HPLC) assay (Mancinelli et al., 1991).

2.3.2. Stability in contact with simulated gastric and intestinal fluids

An amount of 600 mg of MED15 in 200 ml of simulated gastric fluid (A) were shaken for 3 h at 37°C, neutralized with NaOH 1N and freeze-dried. Half of the pellet thus obtained was dissolved in 200 ml of intestinal fluid (B) and, after 3 h of shaking at 37°C, again freeze-dried. Samples were taken every 15 min and identification was performed by thin layer chromatography (TLC) (eluent CH_2Cl_2/CH_3OH 95:5), and detected by ultraviolet (UV), H_2SO_4 2N and I_2.

(A) Simulated gastric fluid (USP23): 2.0 g NaCl and 3.2 g pepsin were dissolved in 7 ml of HCl and distilled water added to a volume of 1000 ml (pH ca. 1.2).

(B) Simulated intestinal fluid (USP23): 6.8 g of KH_2PO_4 were dissolved and mixed in 250 ml distilled water, to which 190 ml NaOH 0.2N and 400 ml distilled water were added. Then, 10.0 g pancreatin were later added, mixed thoroughly, and pH of the resulting solution adjusted to 7.5 ± 0.1 with NaOH 0.2N. Lastly, distilled water was added to a volume of 1000 ml.

2.4. MED15 tissue levels in the gastric and intestinal wall

Fasted or fed animals were treated per os with a single dose of MED15 (100 mg/kg) (the maximum dose used in pharmacology) in CM-cellulose 1%. Groups of six animals were euthanized by cervical dislocation at various times for the determination of MED15 and its metabolites in the mucosa of different anatomical districts as follows: stomach wall = 0.25, 0.5, 1, 1.5, 2 and 3 h; small intestinal wall = 0.5, 1, 3, and 5 h; large intestinal wall = 1, 3, 5 and 24 h. The samples were stored at −20°C after collection until the assay. The tissues, washed in phosphate buffer pH 7.4, were homogenized in the same buffer (tissue-buffer ratio 1:5), and extracted by the same procedure used for plasma.

2.5. Prostaglandin E_2 levels in rat gastric tissue

Rats randomized into groups of 5–8 animals/group and fasted for 18 h were orally administered with MED15 (25, 50 and 100 mg/kg) and tolmetin (15.25, 30.5 and 61 mg/kg) in CM-cellulose 1% (10 ml/kg) or vehicle, for 4 consecutive days. Four hours after the last administration, the animals were euthanized with urethane and the stomachs removed. The stomachs were weighed and homogenized with 2 ml of chilled ethyl alcohol (containing indomethacin 100 μM); after 1 h, samples were centrifuged at 3000 rpm for 10 min at 4°C, the supernatant collected and

again extracted with 2 ml of chilled ethyl alcohol. The collective supernatants were dessicated and the residue dissolved in 2 ml of distilled water; after 1 h, they were extracted on C-18 columns, and eluted with methyl alcohol. The eluate was dessicated and dissolved in assay buffer for radioimmunoassay prostaglandin E_2 determination.

2.6. In vivo gastric acid secretion

Gastric acid inhibition, already determined in vitro (Tubaro et al., 1995) was verified in an in vivo model according to a modified Leithold method (Leithold et al., 1984). Animals fasted for 18 h prior to treatment and divided into groups of 6–14/group, were treated per os with MED15 25–50–100 mg/kg or tolmetin 61 mg/kg (10 ml/kg) suspended in CM-cellulose 1%; the controls received the same volume of vehicle. Forty-five minutes after drug administration, the animals were anaesthetized with urethane (1.25 g/kg i.p.), and the oesophagus was ligated close to the stomach: an incision was made in the forestomach into which a polyethylene cannula was inserted and tied; a second cannula was inserted through the pyloric sphincter. The stomach was perfused with saline (60 ml/h) at 37°C by Masterflex pump; the perfusate was collected after 15 min and titrated to pH 7.0 with NaOH 10^{-2} M to determine the basal acidity.

Histamine, 30 μmol/kg/h (submaximum dose experimentally demonstrated to stimulate gastric acid secretion), was then administered 1 h after MED15 or tolmetin by continuous intravenous infusion (6 ml/h) for the duration of the experiment (120 min = time required to plateau), continuing gastric perfusion with saline and collecting the perfusate every 15 min for the titration; gastric acidity was expressed as μEq H^+/15 min.

To study the effect of MED15 on peptone-stimulated gastric acid production, the animals were treated with the drug and the stomachs prepared as described above. One hour after MED15 or tolmetin, a peptone solution (Difco Micro Inoculum Broth diluted 1:10 at pH 6.6 and sterilized) instead of saline, was perfused directly into the stomach (60 ml/h) and the gastric perfusate, collected every 15 min for a total of 60 min, was titrated to pH 7.0 with NaOH 10^{-2} N. Gastric acidity was expressed as μEq H^+/60 min. A 1-h observation period was established since the acid output of the controls peaked after 45 min and began to fall after 60 min.

2.6.1. Effect of receptor antagonists on peptone-stimulated gastric secretion

The MED15 mechanism of action was also studied in the experimental method of peptone-stimulated gastric secretion described above using various receptor antagonists: capsazepine, CGRP-(8–37) and diphenhydramine (8 rats/group).

(a) Subcutaneous capsazepine (77 μmol/kg, the dose capable of reversing capsaicin action, as in Perkins and Campbell, 1992), was given simultaneously with an oral administration of MED15 (100 mg/kg). Gastric peptone infusion was begun 60 min after treatment and continued for 1 h.

(b) CGRP-(8–37) (290 μg/kg, the dose experimentally demonstrated to be capable of reversing calcitonin gene-related peptide (CGRP) action) (Kato et al., 1994) was delivered by continuous infusion through the femoral vein at a rate of 6 ml/h for 90 min; simultaneously with beginning of infusion, MED15 (100 mg/kg) was administered orally; gastric perfusion with peptone was begun 60 min after the treatment with MED15 and continued for 1 h.

(c) Diphenhydramine (5 mg/kg i.m.) was given 30 min after an oral administration of MED15 (100 mg/kg). Gastric perfusion with peptone was begun 60 min after MED15 administration and continued for 1 h.

2.7. Evaluation of gastric bicarbonate secretion

Bicarbonate levels were evaluated on samples of gastric perfusate in histamine-stimulated gastric secretion, following the method described for acid secretion, and using groups of 10 rats each.

The gastric perfusate (≅ 15 ml), dripped on NaOH 1 M, was collected every 15 min in a 20-ml hermetically sealed vial. An amount of 200 μl HCl 37% were added to the samples and these were incubated at 35°C for 15 min. Bicarbonate levels were evaluated by injecting 0.5 ml of the headspace of samples into a gas-chromatograph (Fractovap, Carlo Erba) fitted with a thermal conductivity detector (TCD).

Analytical conditions: stainless PORAPAC Q 6 ft × 1/8 column; oven temperature = 50°C. Carrier gas = helium 0.5 kg/cm^2. Chromatographic integrator = Shimadzu CR3-A.

2.8. Evaluation of gastric lesions

The animals entered in the experiment, randomized and divided into groups of 6–10 rats each, were fasted for 18 h prior to treatment, with access to water ad libitum. They were re-fed 1 h after drug administration. The animals received MED15 orally (100 mg/kg) suspended in CM-cellulose 1% or vehicle; 30 min later diphenhydramine (5 mg/kg i.m.) was administered to a group of the MED15-treated animals and to a group of vehicle-treated animals. All treatments were administered for 4 consecutive days. Four hours after the last administration, the animals were euthanized by ether overdose, the stomachs removed, rinsed with 10 ml of saline, and immersed in 1% formalin. They were later opened along the greater curvature and the mucosa examined for lesions. Each lesion was graded according to size: (1) ≦ 1 mm; (2) 1–2 mm; (3) ≧ 2 mm.

The sum total was divided by 10 to obtain the ''erosion index'' (Main and Whittle, 1975).

2.9. Prevention of indomethacin-induced gastric lesions

Rats fasted overnight and divided into groups of 7–11 animals/group, were administered a single oral dose of MED15 (25–50–100 mg/kg) or tolmetin (15.25–30.5–61 mg/kg) suspended in CM-cellulose 1%; the controls received the same volume of vehicle (5 ml/kg). One hour after administration, the animals were treated with 20 mg/kg indomethacin, a gastric ulcer-inducing dose (Maggi et al., 1987), per os and euthanized 3 h later by cervical dislocation. The stomachs were examined as described above.

2.9.1. Indomethacin-induced gastric lesions in the presence of receptor antagonist of CGRP (CGRP-(8–37))

CGRP-(8–37) (750 μg/kg, a dose which exceeds the dose normally used in order to ensure complete blockage of CGRP receptors) was dissolved in 0.9% NaCl and, using groups of seven rats each, administered into the tail vein (1.5 ml/kg) simultaneously with a single oral dose of MED15 (100 mg/kg). One hour later, the rats were treated orally with 20 mg/kg of indomethacin, and, after 3 h, the stomachs were removed and examined according to the method described above.

2.10. Statistical analysis

All data are expressed as mean ± S.E.M. of n values. Results were evaluated using One-way analysis of variance (ANOVA) followed by Bonferroni test where necessary, or Mann–Whitney U-test. Significant P values: $< 0.05^{*}$, $< 0.01^{**}$, $< 0.001^{***}$.

3. Results

3.1. Stability of MED15 in contact with biological fluids

In simulated gastric and intestinal fluid MED15 is stable for > 3 h. When in contact with rat plasma, MED15 was quickly hydrolized to its first metabolite [1-methyl-5-(4-methylbenzoyl)-1*H*-pyrrole-2-acetyl]-glycine (MED5), which was stable for up to 3 h after incubation at 37°C, ranging from 85.8% at 2 min to 81.3% at 3 h. During this period, its second metabolite, McN-2559, was produced from MED5 (Table 1).

3.2. Gastrointestinal MED15 levels

Tissue concentrations of MED15 and its metabolites [MED5, McN-2559 and MCPA (1-methyl-5-(4-carboxybenzoyl)-1*H*-pyrrole-2-acetic acid)] in the walls of the stomach, and small and large intestines of the rat are reported in Table 2. Results are in favour of a persistent presence of the unmodified product throughout the gastroenteric wall; peak concentration gradient from stomach to small and large intestine was found. Direct absorption of the MED15 molecule by gastric mucosa is affected by drug concentration at mucosal level: the presence of the molecule in the gastric wall decreases by 57.3 ± 5.81% when food is allowed ad libitum, probably due to drug dilution in food.

3.3. Evaluation of gastric acid secretion

Gastric acid secretion was studied in vivo using a perfused stomach preparation of anaesthetized rats stimulated with various agonists. The effect of MED15 and of tolmetin (McN-2559) was evaluated at equimolecular dosages; tolmetin was chosen as reference compound because it is both a metabolite of amtolmetin guacyl and an NSAID used in therapy. Oral administration of MED15 at 50–100 mg/kg produced marked and statistically significant reduction of the gastric acid secretion induced by histamine (30 μmol/kg/h); while at 25 mg/kg inhibition of gastric acid secretion was not significant compared to controls. Two hours from thrust-off of histamine infusion, the inhibitions of acid secretion, over a period of 15 min, were 20.2%, 67.8% and 80.7% respectively for MED15 at 25, 50 and 100 mg/kg (Fig. 1). Tolmetin administered at 61 mg/kg (equimolecular dose to 100 mg/kg MED15) induced a slight, but not statistically significant, increase in gastric acid output in the 2-h time lapse from thrust-off of

Table 1
Stability in contact with plasma (in vitro)
MED5 = [1-methyl-5-(4-methylbenzoyl)-1*H*-pyrrole-2-acetyl]-glycine.
McN-2559 (tolmetin) = 1-methyl-5-(4-methylbenzoyl)-1*H*-pyrrole-2 acetic acid.

	% distribution of MED15 and its metabolites on total recovery					
	0′	2′	10′	30′	60′	180′
MED15	34.3	0.7	–	–	–	–
MED5	60.2	85.8	85.2	84.8	83.3	81.2
McN-2559	5.5	13.5	14.8	15.2	16.7	18.8

Table 2
MED15 and its metabolites tissue levels in the rat following administration of a 100 mg/kg oral dose
Values are expressed in μg/g of wet tissue ± S.E.

In the wall of the stomach

Compound	0.25 h	0.5 h	1 h	1.5 h	2 h	3 h
MED15	25.8 ± 5.6	152.8 ± 46.7	144.7 ± 43.2	53.0 ± 10.2	24.4 ± 10.6	27.0 ± 14.4
MED5	13.8 ± 2.3	17.9 ± 5.02	44.9 ± 7.2	10.6 ± 2.1	2.3 ± 0.8	4.5 ± 0.9

In the wall of the small intestine

Compound	0.5 h	1 h	3 h	5 h
MED15	11.0 ± 2.1	23.8 ± 5.7	28.5 ± 13.0	14.6 ± 9.2
MED5	10.8 ± 1.9	29.1 ± 5.6	41.7 ± 6.1	22.4 ± 5.3
McN-2559	3.25 ± 0.9	2.6 ± 0.5	7.6 ± 0.6	3.9 ± 0.3
MCPA	–	–	4.0 ± 0.6	4.0 ± 0.5

In the wall of the large intestine

Compound	1 h	3 h	5 h	24 h
MED15	2.75 ± 0.8	8.9 ± 4.2	12.7 ± 3.9	1.1 ± 0.4
MED5	1.97 ± 0.24	13.4 ± 7.7	21.2 ± 7.1	0.8 ± 0.2
McN-2559	0.93 ± 0.46	3.0 ± 0.9	7.2 ± 1.6	1.9 ± 0.2
MCPA	–	–	5.0 ± 0.7	5.0 ± 0.9

histamine infusion. Acid output of the controls rose from 9.2 ± 3.1 μEq H^+/15 min ($T = 0$) to 37.0 ± 7.7 μEq H^+/15 min after 60 min and to 58.9 ± 9.6 μEq H^+/15 min after 120 min of stimulation. Following treatment with Med15, 25 mg/kg acid output at $T = 0$ was 11.2 ± 3.5 μEq H^+/15 min, after 60 min of stimulation was 26.0 ± 6.1 μEq H^+/15 min, and at the end of the stimulation period (120 min) was 47.1 ± 7.5 μEq H^+. The animals treated with MED15 at a dose of 50 mg/kg showed only a slight increase in acid output compared to the basal levels (3.8 ± 1.0 μEq H^+/15 min) in the first 60 min from stimulation (12.5 ± 5.2 μEq H^+/15 min) and for the remaining period of observation the acid increase gradually became more contained (19.0 ± 5.4 μEq H^+/15 min after 120 min). Following administration of 100 mg/kg MED15, acid secretion under stimulation passed from basal values of 7.9 ± 2.2 μEq H^+/15 min to 9.6 ± 3.2 μEq H^+/15 min after 60 min, and 11.4 ± 3.4 μEq H^+/15 min after 120 min. Animals treated with tolmetin did not show any significant change compared to controls; acid output at $T = 0$ was 17.7 ± 4.3 μEq H^+/15 min, 41.0 ± 7.1 μEq H^+/15 min after 60 min, and 58.4 ± 9.1 μEq H^+/15 min after 120 min of stimulation. These data show similar (but not identical) effects between the two higher doses of MED15; there is no apparent close dose-effect correlation, while at 25 mg/kg the inhibition is very slight and not significant, which would suggest a receptor saturation process underlying the inhibitory effect.

Following stomach perfusion with peptone, a dose-related reduction in μEq H^+ was also seen in animals treated with MED15. Total acid output over 1 h infusion was 22.66 ± 3.64 μEq H^+ for the controls and 18.55 ± 2.17, 15.08 ± 4.99 and 7.72 ± 2.23 μEq H^+ following MED15 treatment at 25, 50 and 100 mg/kg respectively

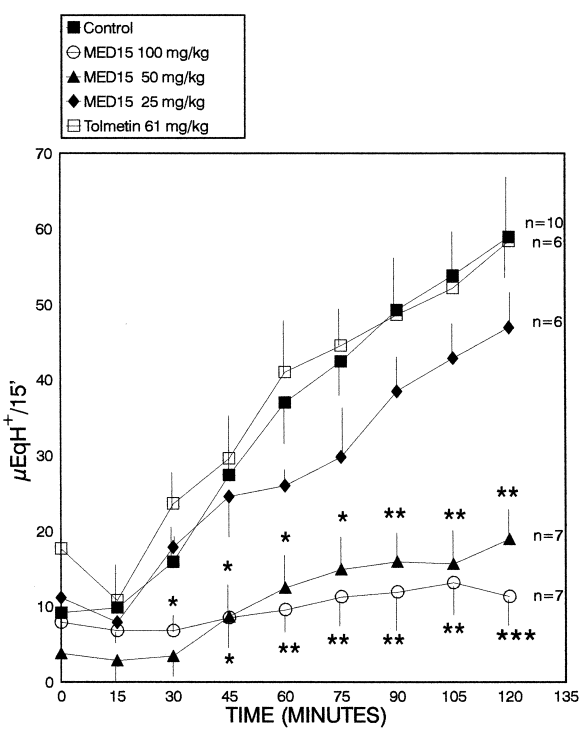

Fig. 1. Effect of MED15 on histamine-induced gastric acid secretion in anesthetized rats. MED15 (25, 50 and 100 mg/kg) and tolmetin (61 mg/kg) were administered orally 1 h before starting i.v. histamine infusion (30 μmol/kg/h). Data expressed as mean ± S.E. values from 6 to 10 rats. Statistical analysis: ANOVA. n.s.; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

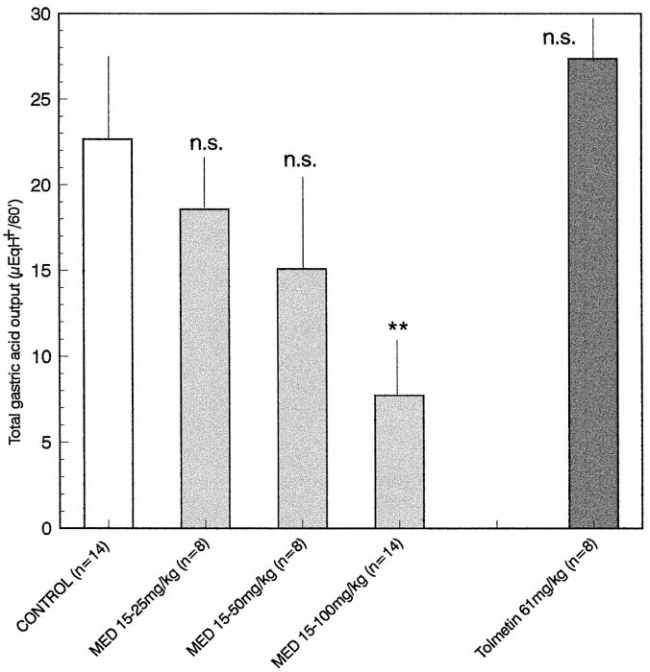

Fig. 2. Effect of MED15 on peptone-induced gastric acid secretion in anesthetized rats. MED15 (25, 50, 100 mg/kg) and tolmetin (61 mg/kg) were administered orally 1 h before starting peptone stomach perfusion. Data expressed as mean ± S.E. values from 8 to 14 rats. Statistical analysis: ANOVA. $^{**}P < 0.01$.

(Fig. 2) with an inhibition of gastric acidity of 18.1%, 33.4% and 67.3%; statistical significance was obtained only at the highest dose. Basic gastric acid secretion was unaffected by pretreatment with MED15 in all of the experiments. Following a 61 mg/kg oral dose of tolmetin, total acid output was 27.35 ± 2.45 μEq H^+, showing an increase of 20.7%.

3.4. Evaluation of gastric bicarbonate secretion

Gastric bicarbonate secretion was studied in animals treated with MED15 (100 mg/kg) and stimulated with histamine (30 μmol/kg/h) for 2 h: total HCO_3^- output was 11.95 ± 0.6 μmol for controls and 21.22 ± 2.95 μmol for MED15-treated animals ($P < 0.05$), showing an increase of 77.6% (Fig. 3).

3.5. Evaluation of gastric prostaglandin E_2

Gastric prostaglandin E_2 levels were determined in the gastric mucosa of rats treated per os with MED15 or tolmetin at various dosages (Table 3). A dose-related fall in prostaglandin levels was noted in all groups when compared to controls. In the animals treated with MED15 at 25, 50 and 100 mg/kg, gastric PGE_2 levels were 28.06 ± 5.27, 19.19 ± 4.07 and 16.14 ± 4.48 ng/g tissue, respectively, and 47.13 ± 6.11 ng/g tissue in the controls. Gastric PGE_2 levels following treatment with tolmetin at 15.25, 30.5 and 61 mg/kg were 27.19 ± 4.16, 21.64 ± 6.62 and 17.18 ± 1.88 ng/g tissue, respectively.

All of the treatments demonstrated statistical significance compared to controls.

The data obtained demonstrate that both products examined produce a fall in gastric prostaglandin levels in a similar manner when administered by equimolecular doses.

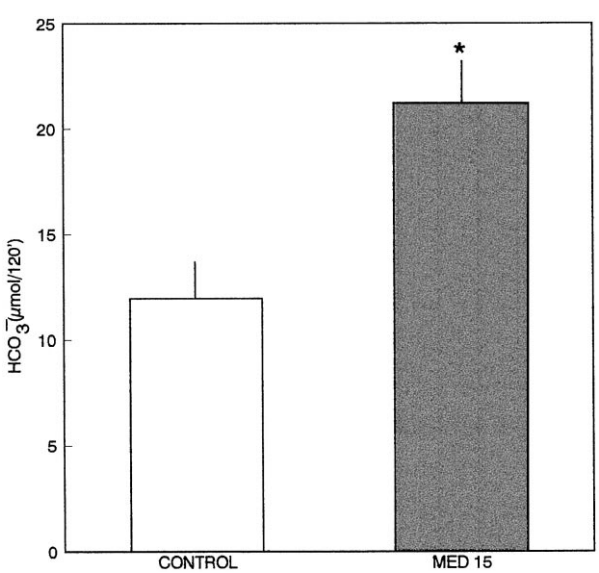

Fig. 3. Effect of MED15 on histamine-induced gastric bicarbonate secretion in anesthetized rats. MED15 (100 mg/kg) was administered orally 1 h before starting i.v. histamine infusion (30 μmol/kg/h). Data expressed as mean ± S.E. values from 10 rats. Statistical analysis: ANOVA. $^{*}P < 0.05$.

Table 3
Inhibition of MED15 and of tolmetin on gastric PGE_2 in the rat
MED15 and tolmetin administered for 4 consecutive days. The stomachs were removed 4 h after the last administration.

Groups	No. of animals	Dose (mg/kg os)	PGE_2 (ng/g tissue ± S.E.)	% inhibition	*P*
Controls	7	–	47.13 ± 6.11	–	–
MED15	8	100	16.14 ± 4.48	65.8	0.001
	5	50	19.19 ± 4.07	59.3	0.006
	7	25	28.06 ± 5.27	40.5	0.036
Tolmetin	7	61	17.18 ± 1.88	63.5	0.0001
	7	30.5	21.64 ± 6.62	54.1	0.015
	8	15.25	27.19 ± 4.16	42.3	0.016

3.6. Effect of MED15 on indomethacin-induced gastric damage

Oral administration of indomethacin dose-dependently damages gastric mucosa; the effect of pretreatment with MED15 and tolmetin on indomethacin-induced gastric lesions was evaluated. An oral dose of 20 mg/kg of indomethacin induces severe mucosal damage (erosion index = 6.77 ± 0.51). MED15 produces a dose-related improvement in indomethacin-induced damage, producing erosion indexes of 6.25 ± 1.19, 5.30 ± 0.23 and 4.66 ± 0.65 at 25, 50 and 100 mg/kg, respectively. On the contrary, under the same experimental conditions, tolmetin induces deterioration that does not appear to be dose-related. In fact, the erosion indexes of tolmetin at 15.25, 30.5 and 61 mg/kg are 8.91 ± 0.46, 8.94 ± 0.66 and 9.27 ± 0.73, respectively, always statistically different from controls (Fig. 4).

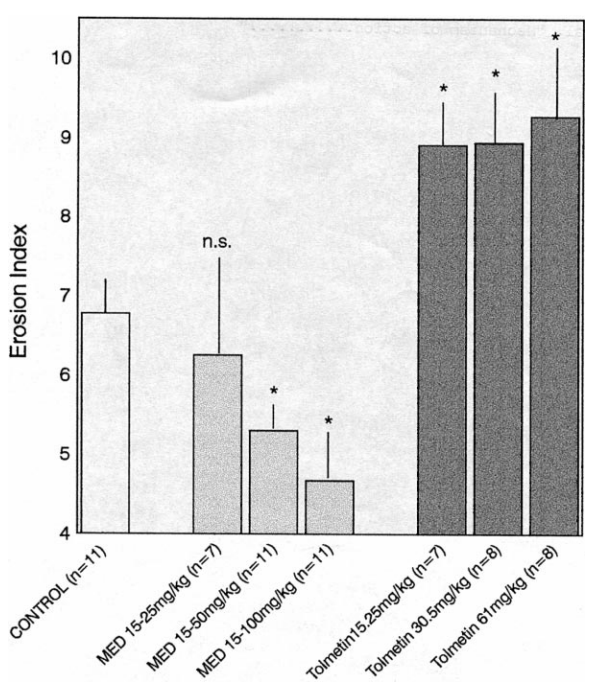

Fig. 4. Effect of MED15 on indomethacin-induced gastric lesions in rats. MED15 (25, 50 and 100 mg/kg) and tolmetin (15.25, 30.5 and 61 mg/kg) were administered orally 1 h before indomethacin (20 mg/kg per os). Stomachs were removed 4 h after MED15 administration. Data expressed as mean ± S.E. values from 7 to 11 rats. Statistical analysis: Mann–Whitney *U*-test. $^*P < 0.05$.

3.7. Interference of various receptor antagonists on gastric effects of MED15

The MED15 molecular structure is suggestive of its mechanism of action. In fact, vanillic radicals such as the one contained in the structure of MED15 are known to stimulate capsaicin receptors that control gastric function. To verify the impact of MED15 on these receptors, animals were treated with MED15 and capsazepine (specific receptor antagonist of capsaicin) in order to investigate their effect on gastric acid secretion. Significant loss of MED15 antisecretory activity was found. Total acid output produced in the peptone-perfused stomach over 60 min gave the following results: control, 17.9 ± 3.84 μEq H^+; MED15, 5.67 ± 1.45 μEq H^+; capsazepine, 15.90 ± 2.3 μEq H^+; MED15 + capsazepine, 18.71 ± 2.1 μEq H^+ (Fig. 5). Additional indirect demonstration of the impact of MED15 on capsaicin (vanilloid) receptors is the interference observed with histamine H_1 receptor antagonists: interference with capsaicin receptors by drugs such as diphenhydramine and pyrilamine is reported by various AA (Wallace et al., 1992; Mathison and Davison, 1995). Fig. 6 illustrates the diphenhydramine inhibition of the antisecretory effect of MED15 (total acidity over 60 min: control, 20.11 ± 5.01 μEq H^+; MED15, 6.5 ± 1.62 μEq H^+; diphenhydramine, 17.19 ± 6.94 μEq H^+; MED15 + diphenhydramine, 17.87 ± 1.58 μEq H^+). The gastrolesive effects of MED15 arose in the presence of diphenhydramine as well (Fig. 7). The mechanism of action was therefore found to involve the capsaicin receptors, which in their turn are known to strictly interact with CGRP receptors in controlling gastric function (Clague et al., 1985; Lippe et al., 1989; Peskar et al., 1993; Ren et al., 1993). To evaluate the possible impact of MED15 on CGRP receptors, CGRP-(8–37) (a specific CGRP receptor antagonist) was used. When CGRP-(8–37) was co-administered with MED15 to rats, the strong reduction of peptone-induced gastric acid secretion was lost. Fig. 8 shows that gastric acidity in MED15 and CGRP-(8–37)-treated

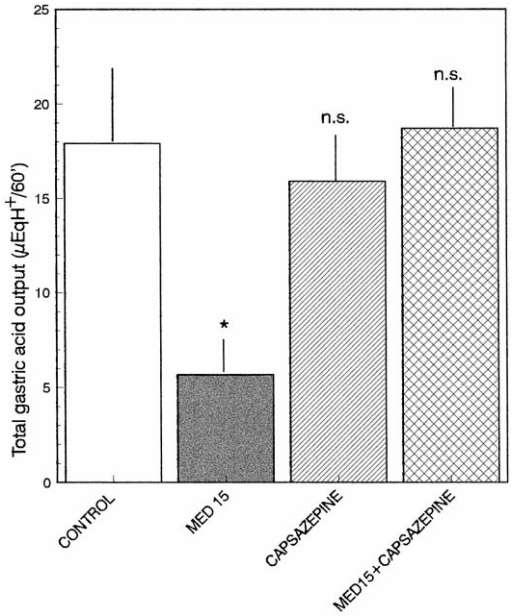

Fig. 5. Effect of MED15 on peptone-induced gastric acid secretion in capsazepine-treated rats. MED15 (100 mg/kg p.o.) and capsazepine (77 μmol/kg s.c.) were administered simultaneously 1 h before starting peptone stomach perfusion. Data expressed as mean ± S.E. values from eight rats. Statistical analysis: ANOVA and Bonferroni tests. $^{*}P < 0.05$; n.s.; MED15 + capsazepine vs. capsazepine n.s.

rats is similar to the levels of the rats treated with CGRP-(8–37) only (30.43 ± 5.4 and 28.68 ± 5.93 μEq H^+, respectively), while in the same experiment, control was

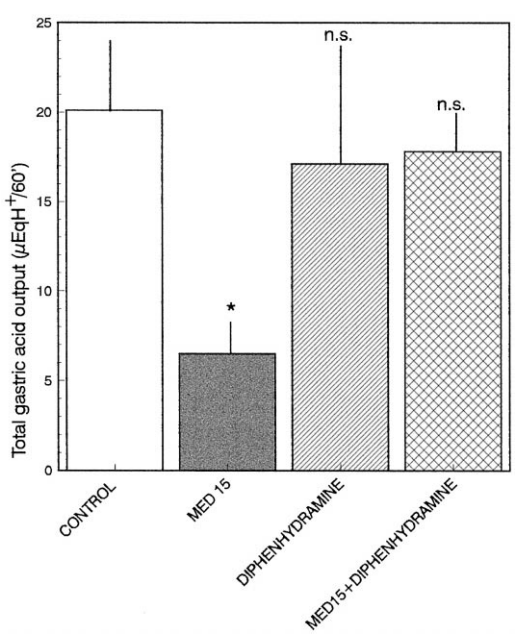

Fig. 6. Effect of MED15 on peptone-induced gastric acid secretion in diphenhydramine-treated rats. Diphenhydramine (5 mg/kg i.m.) was administered 30 min after MED15 (100 mg/kg p.o.) and peptone stomach perfusion started after 30 min. Data expressed as mean ± S.E. values from eight rats. Statistical analysis: ANOVA and Bonferroni tests. $^{*}P < 0.05$; n.s.; MED15 + diphenhydramine vs. dephenhydramine n.s.

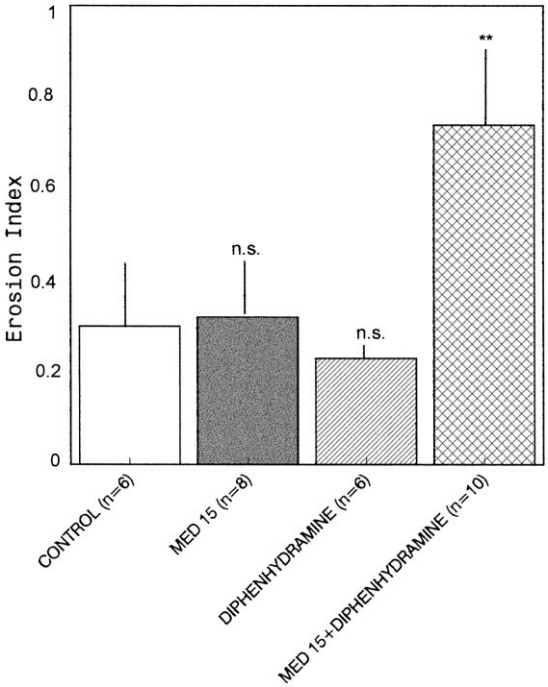

Fig. 7. Interference of diphenhydramine on gastroprotective action of MED15 in the rat. MED15 (100 mg/kg p.o.) was administered 30 min before diphenhydramine (5 mg/kg i.m.) for 4 consecutive days; erosion index was determined 4 h after the last administration. Data expressed as mean ± S.E. values from 6 to 10 rats. Statistical analysis: Mann–Whitney *U*-test. $^{**}P < 0.01$; n.s.; MED15 + diphenhydramine vs. diphenhydramine. $^{**} < 0.01$.

18.25 ± 4.12 μEq H^+, and MED15 alone was 5.96 ± 1.8 μEq H^+. Confirmation of the involvement of CGRP in the

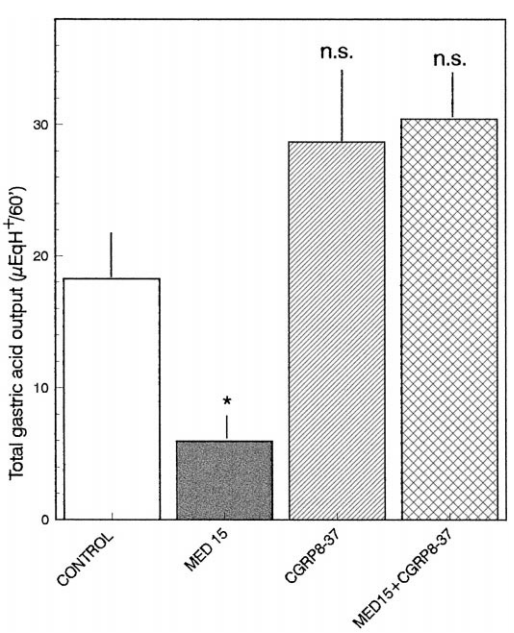

Fig. 8. Effect of MED15 on peptone-induced gastric acid secretion in CGRP-(8–37)-treated rats. MED15 (100 mg/kg p.o.) and CGRP-(8–37) (290 μg/kg infused i.v. for 90 min) were administered simultaneously 1 h before starting peptone stomach perfusion. Data expressed as mean ± S.E. values of eight rats. Statistical analysis: ANOVA and Bonferroni tests. $^{*}P < 0.05$; n.s.; MED15 + CGRP-(8–37) vs. CGRP-(8–37) n.s.

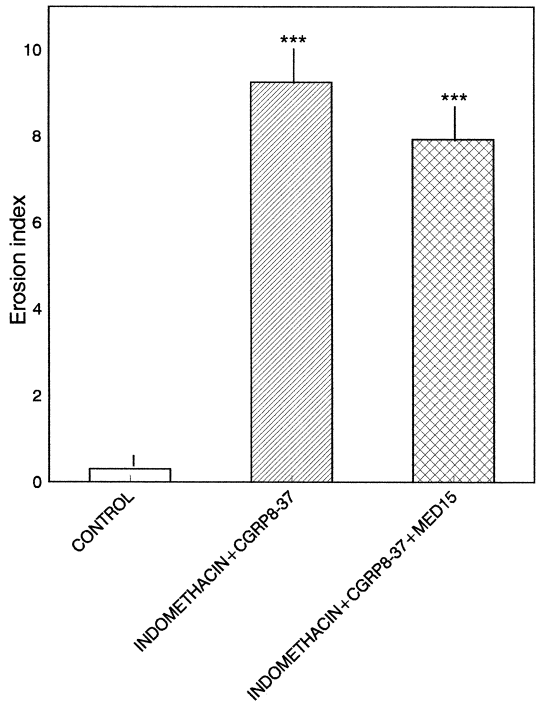

Fig. 9. Effect of MED15 on indomethacin-induced gastric lesions in CGRP-(8–37)-treated rats. MED15 (100 mg/kg p.o.) and CGRP-(8–37) (750 μg/kg i.v.) were administered simultaneously 1 h before indomethacin (20 mg/kg p.o.), controls received only vehicle. Stomachs were examined 4 h after MED15 administration. Data expressed as mean ± S.E. values from seven rats. Statistical analysis: Mann–Whitney *U*-test. $^{***}P < 0.001$. MED15 + indomethacin + CGRP-(8–37) vs. Indomethacin + CGRP-(8–37) n.s.

gastroprotective mechanism of MED15 was found in the model of indomethacin-induced gastric lesions: CGRP-(8–37) abolished the protective effect of MED15 (erosion index MED15 + CGRP-(8–37) = 7.92 ± 0.9, erosion index CGRP-(8–37) = 9.25 ± 1.23) (Fig. 9).

4. Discussion

Currently available NSAIDs effectively control pain and inflammation but they can also induce various degrees of gastrointestinal toxicity. They are damaging to the gastric and intestinal mucosa through down-regulation of those prostaglandins delegated to gastric and intestinal mucosa protection (Hudson et al., 1992). The most common and serious side effects are gastrointestinal ulceration, perforation, and bleeding. Moreover, risk of the development of ulcers is significantly higher among subjects with a previous history of such events or under concomitant glucocorticoid therapy, in the elderly and the female gender (Fries et al., 1989). It is known that NSAIDs are cause of gastric mucosal damage through two mechanisms (Schoen and Vender, 1989; Wallace, 1997): direct topical effect that determines variation in membrane permeability, and systemic inhibition of natural mucosal protection through inhibition of cyclooxygenase activity of the gastrointestinal mucosa, which up-regulates gastric acid secretion and down-regulates synthesis and secretion of gastric mucus and bicarbonate, together with mucosal blood flow (Rainsford, 1985; Scarpignato, 1995). In clinical practice, symptoms range from modest gastric upset to life-threatening gastric and/or intestinal ulceration in relation to the patient's condition and the duration of treatment. Pre-existing conditions such as NSAID-intolerance, gastritis and gastroenteric ulcers are recognized exacerbating factors.

An ideal antiphlogistic agent should be capable of controlling inflammation through adequate prostaglandin inhibition, avoiding renal and gastroenteric side effects. This objective is one of the major unsettled issues of therapy, although many attempts have been made and partial successes obtained worldwide (Wallace, 1997).

Gastric acid-inhibiting agents are normally associated with NSAID therapy due to the well-recognized up-regulation of acid production caused by these drugs, in order to contain the risks of gastric damage: ulcer prevention is normally obtained by treatment with histamine H_2 receptor antagonists, proton pump inhibitors, or gastroprotective prostaglandins (Earnest, 1990; Howden and Holt, 1991; Hayllar et al., 1992; Yeomans et al., 1992).

MED15 demonstrated good anti-inflammatory, antipyretic and analgesic properties, similar to reference NSAIDs, in several pharmacological models [carrageenan-induced paw oedema (Tubaro et al., 1995; Lo Giudice et al., 1998), acetic acid-induced peritonitis, adjuvant arthritis, and others (William Harvey Internal Report)] and in clinical trials (Petazzi et al., 1990a,b; Alicicco et al., 1995; Tavella and Ursini, 1997), showing prolonged pharmacological effect and lack of gastrointestinal side effects. MED15 demonstrated lack of gastric damaging action in the rat also following administration of a very high dosage (up to 300 mg/kg) (Tubaro et al., 1995; Pisano et al., 1999); dosages exceeding this level were not used because they are considered too far removed from the therapeutic range. Pharmacokinetic studies carried out following oral administration (parenteral administration of the drug is not possible due to its total insolubility in water and in the main solvents usable for in vivo administration) demonstrated high levels of unmodified MED15 both in the mucosa of the gastric wall and of the small and large intestinal walls for extended periods of time with consequent slow absorption from the gastroenteric mucosa into the bloodstream: this drug reservoir in the gastroenteric walls could possibly explain the extended pharmacological effect of the drug. The results of the in vitro stability studies indicate that MED15 is stable in gastric and intestinal fluids, whereas it is rapidly hydrolized in plasma to MED5. This appears to demonstrate that the unmodified drug concentrations in the gastrointestinal walls are attributable to direct drug absorption well prior to its transit to the bloodstream.

The effect of MED15 on the gastric mucosa was studied in comparison with its metabolite tolmetin, an NSAID used in clinical practice; there was extreme diversity of performance between the two products towards both gastric acid secretion and gastric damage, with MED15 demonstrating surprising gastroprotective properties for an NSAID.

Attempts to explain the mechanism of action of MED15 led to the evaluation of its effect on cyclooxygenases 1 and 2: cyclooxygenase 1 is constitutive and its inhibition is responsible for the anti-inflammatory effects as well as the side effects; cylooxygenase-2 is inducible and its inhibition is responsible for the anti-inflammatory effects only; consequently, an NSAID capable of preferentially blocking cyclooxygenase-2 is claimed to reduce adverse effects on the gastrointestinal tract (Arai et al., 1993; Kargman et al., 1996). However, Schmassmann reported that a selective inhibitor of cyclooxygenase-2 was demonstrated to be injurious to the mucosa (Schmassmann, 1998). More recently, Cryer and Feldman conclude, in an ample survey of clinical results, for a limited sparing effect of cyclooxygenase-2 selective NSAIDs on gastric cyclooxygenase activity (Cryer and Feldman, 1998).

In vitro experiments carried out by the William Harvey Institute of London using bovine aortic endothelial cells (containing exclusively cyclooxygenase-1 receptors) and LPS-activated J774.2 macrophages (containing exclusively cyclooxygenase-2 receptors) do not appear to indicate preferential MED15-mediated inhibition of cyclooxygenase-2, as confirmed by the strong inhibition of gastric prostaglandin E_2, similar to the inhibition determined by tolmetin: at this juncture, alternative mechanisms were necessarily considered. In vitro and in vivo gastric acid secretion inhibition was at first presumed as a possible histamine H_2 receptor antagonism, but the lack of MED15 activity on the histamine H_2 receptors of the isolated guinea-pig atrium (data not shown), quickly rejected this hypothesis.

Capsaicin receptors were then considered, following on the presence in the chemical structure of MED15 of a vanillic moiety (Fig. 10) known to interact with capsaicin receptors (Szallasi and Blumberg, 1990).

The anti-secretive effect of MED15 abolished by capsazepine (a specific capsaicin receptor antagonist) in an in vivo rat model is a clear demonstration of the impact of MED15 on capsaicin receptors; up-regulation of gastric bicarbonate output is indirect proof of capsaicin-receptor involvement (Takeuchi et al., 1993): capsaicin-sensitive sensory neurons have been shown to be involved in basal and acid-induced bicarbonate secretion. Further confirmation of the impact of MED15 on capsaicin receptors was the down-regulation of the antisecretive effect following co-administration with histamine H_1 receptor antagonists, which interfere with these receptors (Wallace et al., 1992; Mathison and Davison, 1995). Although MED15 impacts on vanilloid receptors, it nevertheless does not demonstrate

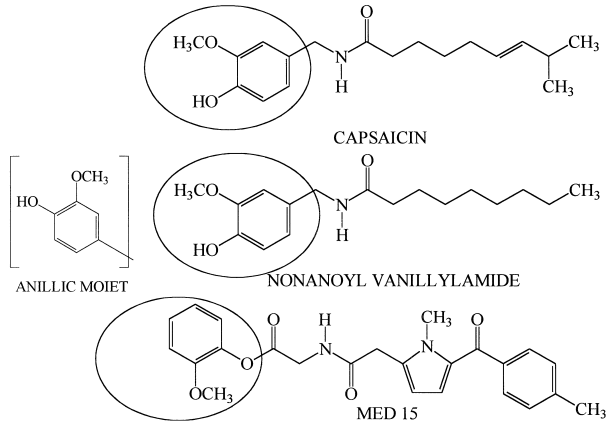

Fig. 10. Presence of a vanillic moiety in the chemical structure of MED15, capsaicin and nonanoyl vanillyllamide.

the revulsive effects typical of capsaicin since its structure contains only the vanilloid moiety and not the molecular pole responsible for the irritative effect (Yeh et al., 1993; Wu et al., 1995, 1996) which can be suppressed (nonanoyl vanillylamide) or potentiated (resiniferatoxin).

Literature data indicate that capsaicin-sensitive nerves play a role in gastric defence mechanisms which counteract the adverse effects of ulcerogens (Maggi, 1990). Capsaicin receptors are present throughout the gastrointestinal mucosa (Buck and Burks, 1986) and may be involved in gastroenteric motility regulation, acid secretion and blood flow, or mucosal integrity maintenance against acid and other noxious substances (Holzer et al., 1990a). Capsaicin action on gastric mucosa is prostaglandin-independent since indomethacin pretreatment does not prevent the protection induced by intragastric administration of capsaicin (Holzer et al., 1990b). The gastroprotective action of afferent nerve stimulation by capsaicin is most likely brought about by the local release of a transmitter within the gastric mucosa. Capsaicin-sensitive neurons contain, in fact, a number of vasoactive peptides, including substance P and other tachykinins, CGRP, and vasoactive intestinal polypeptide (VIP) (Green and Dockray, 1988). CGRP is the predominant sensory neuropeptide in the rat gastric mucosa and immunocytochemical studies indicate that CGRP-containing nerves within the myenteric and submucosal plexuses of the rat stomach are derived solely from capsaicin-sensitive primary sensory afferent nerves (Clague et al., 1985). CGRP is a 37-amino acid peptide which inhibits acid secretion and is capable of increasing gastric mucosal blood flow and protecting against ethanol- and aspirin-induced mucosal injury (Lippe et al., 1989). CGRP would thus appear to be a candidate mediator of both the gastric vasodilator and protective effect, with the role of modulator, transmitter, and hormone. To further clarify the mechanism of action of MED15 the specific receptor antagonist of CGRP [CGRP-(8–37)] was used. The suppression of antisecretory and gastroprotective effects was a demonstration that CGRP is the main neuropeptide responsible for

the impact of MED15 on the gastric mucosa, as suggested by the known relationship between capsaicin and CGRP receptors (Holzer et al., 1990b; Kinoshita et al., 1993; Peskar et al., 1993; Ren et al., 1993).

CGRP acts in the stomach both directly, and indirectly through release of nitric oxide (Holzer et al., 1994, 1995); when administered with L-NAME (a specific nitric oxide synthase inhibitor) the gastroprotective effect of MED15 is dramatically reduced (Pisano et al., 1999). This is in favour of nitric oxide as a component of the final protection mechanism offered by MED15 towards the gastric mucosa.

In conclusion, MED15 down-regulates gastric prostaglandin production, as all known NSAIDs do. However, differently from these, the presence of a vanillic moiety in the MED15 molecule induces a gastroprotective effect that offers new exploitation possibilities in the long-term treatment of inflammatory diseases.

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