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Impact on the bowel of amtolmetin guacyl, a new gastroprotective non-steroidal anti-inflammatory drug

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Abstract

Amtolmetin guacyl (MED15) is a new non-steroidal anti-inflammatory drug (NSAID) which shares anti-inflammatory, analgesic and antipyretic activity with the other drugs of the NSAID family but which shows, unexpectedly, strong gastroprotective activity similar to misoprostol. This effect has been attributed to the presence in its molecule of a vanillic moiety responsible for stimulation of capsaicin receptors present throughout the length of the gastrointestinal tract. MED15 shows antispasmodic activity in the bowel against a number of agonists and compares favourably with reference compounds. In in vivo indomethacin-induced rat ileitis, MED15 heals better than 5-aminosalicylic acid and sulfasalazine, as well as down-regulating intestinal wall myeloperoxidase content. In acetic acid-induced colitis in the rat, levels of malondialdehyde were found to be more markedly reduced with MED15 than with 5-aminosalicylic acid. In contrast with the effect in the stomach, MED15 protective effect in the bowel appears to be unrelated to nitric oxide (NO) production. The MED15 enteroprotective effect is related to stimulation of intestinal capsaicin receptors as demonstrated by the loss of protective effect in the presence of capsazepine, a specific receptor antagonist of capsaicin. In conclusion, following the favourable results obtained in animal models and notwithstanding the pharmacological effects typical of an NSAID, MED15 may rationally be proposed for the treatment of various human colitis conditions and Crohn's disease.

Keywords: Amtolmetin guacyl; Intestine; Inflammation; Neuropeptide; (Rat)

1. Introduction

Amtolmetin guacyl (2-methoxyphenyl-1-methyl-5-*p*-methyl benzoyl-pyrrol-2-acetamido acetate) or MED15, introduced in recent years in clinical therapy, showed exceptional gastric tolerance together with anti-inflammatory efficacy comparable with the traditional top non-steroidal anti-inflammatory drugs (NSAIDs) (Ghirardini et al., 1990; Petazzi et al., 1990a,b; Simon et al., 1998; Bianchi Porro et al., 1999; Lanza, 1999; Marcolongo et al., 1999; Montrone et al., 2000; Tubaro et al., 2001).

Studies carried out with MED15 in various animal models demonstrated the absence of any damage to the gastric mucosa, as evaluated by macroscopic examination of the gastric mucosa as well as by gastric wall histology (Tubaro et al., 1995); subsequent pharmacological studies demonstrated a gastroprotective effect of MED15 on indomethacin-induced gastric lesions in the rat (Pisano et al., 1999; Tubaro et al., 2000). The mechanism of this gastric protection was attributed to the presence in the molecule of a vanillic (capsaicin) moiety which, through stimulation of capsaicin receptors, causes neuropeptide (calcitonin gene-related peptide or CGRP) release (Holzer et al., 1990a,b; Kinoshita et al., 1993) and, consequently, nitric oxide (NO) production (Holzer et al., 1993; Chen and Guth, 1995). Capsaicin-like behaviour of MED15 was demonstrated in several studies using either capsazepine, a specific receptor antagonist of capsaicin or, through defunctionalization of sensory neurons by high doses of capsaicin: in both models, the gastroprotective effect was lost (Tubaro et al., 2000 and unpublished data). Confirmation of the protective activity of MED15 was found in healthy human volunteers, in alcohol-induced stomach damage; in this study, a misoprostol-like effect of MED15 was demonstrated using electrogastrography (Riezzo et al., 2001). This result is unsurprising, given the known capsaicin-related inhibition of gastric damage (Evangelista et al., 1987).

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The peculiar effect of MED15 on the gastric mucosa first attracted the authors' attention because of the well-known clinical demand for a NSAID devoid of adverse gastric events.

Furthermore, the pharmacokinetic profile of MED15 demonstrated absorption of the product via the gastroenteric mucosa, where it resides at length as the unmodified molecule together with its metabolites (Tubaro et al., 2000). These data have raised the question of a possible pharmacological effect at the intestinal level, progressing to further specific in vitro and in vivo studies aimed at definition of these characteristics of the drug and the possibility of their exploitation in human bowel disease.

2. Materials and methods

2.1. Animals

Male Wistar rats, 150–300 g b.w. and male guinea pigs 280–320 g b.w. (Nossan, Italy) were used.

The animals were housed under conventional husbandry conditions for at least 1 week in a controlled environment before use. A commercial diet and tap water were provided ad libitum.

Animals were fasted for 18 h before the studies, on wire gratings to avoid coprophagy, and with free access to water.

The animal studies were carried out in conformity with the international guidelines (NIH guide for the care and use of laboratory animals, NIH, Bethesda, MA, USA). Qualified veterinary examination excluded drug-related abnormalities.

2.2. Chemicals

Acetylcholine chloride, bradykinin, histamine dichloride, 5-hydroxytryptamine hydrochloride dissolved in Tyrode's solution; *O*-di-anisidine dihydrochloride and standard myeloperoxidase dissolved in 50 mM potassium phosphate buffer; acetic acid, hexadecyltrimethyl-ammonium bromide, hydrogen peroxide, sodium acetate trihydrate, dimethylsulfoxide (DMSO), potassium sulphate, CM-Cellulose and 1,1,3,3-tetraethoxypropane dissolved in water; 2-thiobarbituric acid in 2 M sodium acetate buffer, butylhydroxytoluene in absolute ethanol; N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) in saline; capsazepine in 50% ethanol; indomethacin dissolved in $NaHCO_3$ 5% for in vivo studies and in DMSO for in vitro studies; *n*-butanol, ethanol, methanol.

All chemicals were purchased from Sigma.

2.2.1. Study drug and reference compounds

Amtolmetin guacyl (MED15) (Medosan Ricerca, Italy), 5-aminosalicylic acid and sulfasalazine (Sigma) dissolved in CM-Cellulose 1% for in vivo studies.

MED15, *N*-[1-methyl-5-(4-methylbenzoil)-1*H*-pyrrol-2-acetyl]-glycine (MED5) (Medosan Ricerca, Italy), nabumetone and tolmetin (Sigma) dissolved in DMSO for in vitro studies.

2.3. Effects on the intestine

2.3.1. Guinea pig isolated ileum

Animals were dispatched by cervical dislocation and exsanguination. Immediately afterwards, an approximate 2.5-cm length of ileum, lying 4–5 cm from the ileocaecal valve, was removed and transferred to a Petri dish of Tyrode's solution at 37 °C. The washed tissue was then mounted in a 100-ml bath containing Tyrode's solution (composition, in mM: NaCl 136.9; KCl 2.7; $CaCl_2$ 1.6; $MgCl_2$ 1.1; NaH_2PO_4 0.5; $NaHCO_3$ 7.7; glucose 5.6) gassed with 95% O_2:5% CO_2 and maintained at 37 °C. The tissue was loaded with 1.0 g and contractions were detected isometrically by a force transducer (mod. 7003 Basile, Italy) equipped with a recording dynamometer (mod. 7050, Basile). Two models of ileum stimulation were performed: single dose and cumulative doses (only for acetylcholine and histamine stimulation). For the single dose experiments, after ileum equilibration for at least 60 min, with replacement of fresh solution every 15 min, test compounds or vehicle (DMSO) were added and incubated for 5 min; agonist was then added and the contractile response of ileum recorded for 3 min. In the cumulative dose experiments, 5 min after test compound or vehicle, agonist was added at gradually increasing doses, without washing out between following doses. In both models, at the end of recording, bathing solution was changed and the ileum washed until restoration of the baseline tension was obtained. After each in vitro testing, the effect of the vehicle (DMSO) was once again determined on agonist-induced contractions.

MED15 was tested at 3.12, 6.25, 12.5, 25, 50 and 100 μM and was compared, in bradykinin-induced response, with tolmetin (100–150 and 200 μM), indomethacin, MED5, nabumetone at 50–100 and 200 μM.

Agonists:
bradykinin: 10^{-7} M
histamine: from 10^{-9} to 10^{-7} M
acetylcholine: from 10^{-9} to 5×10^{-8} M
5-hydroxytryptamine: 10^{-7} M

2.3.2. Indomethacin-induced intestinal inflammation in the rat

Fasted rats (280 ± 20 g) were treated subcutaneously with indomethacin 7.5 mg/kg in sterile $NaHCO_3$ 5% (2.5 ml/kg) for 2 consecutive days (days 0 and 1). MED15, 5-aminosalicylic acid and sulfasalazine 100 mg/kg were administered orally in CM-Cellulose 1% (6 ml/kg) at days −1, 0, 1, 2 and 5. Administration of vehicle to the controls was performed at the same times as the products. Animals were fasted before the administration of substances because the study drug, due to its mechanism of action, should preferably be administered on an empty stomach.

Animals were weighed and dispatched 24 h after the last administration; the small bowel was removed from the pylorus to the ileum–caecal valve and the length measured (standardised tension was reached by fixing a small weight at one end); it was then opened lengthwise, washed with buffered sodium chloride solution (pH 7.4), and dried on filter paper (Wathman no. 1). The "body weight/bowel length" index, estimated to reflect the severity of inflammation, was determined. The length of the small intestine does, in fact, depend on the body weight of the rats (Yamada et al., 1993) and inflammation induces bowel shortening.

Macroscopic damage was scored blind by two observers in accordance with the 0–10 Kullman scale (Kullman et al., 1997) and total number of ulcers counted.

Score:
0 = normal;
1 = hyperemia and/or petechial bleeding;
2 = single mucosal erosion;
3 = single mucosal erosion or ulcer with hyperemic, adhesive or haemorrhagic lesions;
4 = multiple erosions or ulceration, major sites of damage extending < 10 cm along length of bowel;
5–10 = score was increased by 1 for each additional 10 cm of involvement.

Immediately after scoring, the tissues were used for the measurement of myeloperoxidase activity in conformity with Bradley et al. (1982) and Morris et al. (1989).

2.3.3. L-NAME effect in indomethacin-induced intestinal damage

In this experiment, rats of 160 ± 10 g were used. Indomethacin-induced intestinal damage was performed as described above. MED15 100 mg/kg was administered orally at days −1, 0, 1, 2 and 5 simultaneously with L-NAME (NO synthase specific inhibitor) 5 mg/kg s.c. in saline. Controls received vehicle only. Number of ulcers and score were determined.

2.3.4. Capsazepine effect in indomethacin-induced intestinal damage

In this experiment, the same model of indomethacin-induced intestinal damage above described was used.

Capsazepine (77 μmol/kg s.c.) in 50% ethanol and MED15 (100 mg/kg p.o.) were administered simultaneously at days −1, 0, 1, 2 and 5.

Indomethacin (7.5 mg/kg s.c.) was given at days 0 and 1. Controls received vehicle only. Number of ulcers and score were determined 4 h after the last administration.

2.3.5. Myeloperoxidase determination in indomethacin-inflamed ileum

Samples were homogenized (30 s in Ultraturrax) in 0.5% hexadecyltrimethyl-ammonium bromide in 50 mM potassium phosphate buffer (pH 6.0) to give a 50 mg/ml suspension. Aliquots (5 ml) of the suspension were frozen and thawed three times. Following centrifugation (10 000 rpm) for 2 min at 4 °C, 100 μl of the supernatant was mixed with 2.9 ml of 50 mM phosphate buffer containing 0.167 mg/ml of *O*-di-anisidine dihydrochloride and 0.0005% hydrogen peroxide. The myeloperoxidase activity was calculated by determining the change of absorbance at 460 nm of peroxide per minute at 25 °C.

2.3.6. Malondialdehyde determination in acetic acid-induced colon inflammation in the rat

Colitis was induced in fasted rats through administration of 3% acetic acid (1.5 ml/rat) into the lumen of the distal colon, by means of an 8-cm cannula.

MED15 (100 mg/kg p.o.) and 5-aminosalicylic acid (400 mg/kg p.o.) were administered at $T = -48$, -24 and -2 h. The controls received the vehicle only (saline and CM-Cellulose 1%). Twenty-four hours after induction of colitis, the animals were dispatched by cervical dislocation, the colon was removed, washed in saline at pH 7.4 and used for malondialdehyde evaluation. Samples were weighed and homogenized (10% w/v) in saline.

An aliquot (200 μl) of homogenate or 1,1,3,3-tetraethoxypropane solution, representing malondialdehyde external standard, was mixed with 2.0 ml of 0.2% 2-thiobarbituric acid in 2 M sodium acetate buffer (pH 3.5) and 20 μl of 5% butylhydroxytoluene in 99% ethanol. The sample mixture was heated for 45 min in a 95 °C water bath, then cooled under running tap water; following addition of 2 ml *n*-butanol, it was mixed vigorously and centrifuged at 3400 rpm for 15 min. The organic phase was collected and filtered on a 0.45-μm filter for a high-pressure liquid chromatography (HPLC) analysis. Reverse-phase HPLC was performed isocratically using a Perkin-Elmer Series 410 pump, with a Rheodyne 7125 injector (with 20 μl loop) and Perkin Elmer LC 240 fluorescence detector. The separation was carried out with a Bondapack C18 (250 × 4.6 mm) stainless steel column. The mobile phase (water/methanol, 60:40) was filtered at 0.45 μm and degassed under helium stream before use. The 2-thiobarbituric acid–malondialdehyde complex was monitored by fluorescence detection, with excitation at 515 nm and emission at 553 nm.

The injection volume was 20 μl and the flow rate 2.0 ml/min at room temperature. The data were processed by PE Nelson 1022 model.

The quantity of malondialdehyde present in the sample was obtained by comparison between the area of 2-thiobarbituric acid–malondialdehyde complex peak of the chromatogram and the calibration plot (area vs. concentration of malondialdehyde) obtained with malondialdehyde external standard solutions (5–25 nmol in 200 μl).

2.4. Statistical analysis

All data are expressed as mean ± S.E.M. of n values. Statistical evaluation of parametric data was assessed by one-way analysis of variance followed by Student–Newman–Keuls post-hoc test, and values of $P<0.05$ were regarded as significant. Non-parametric data were assessed by Mann–Whitney U test.

3. Results

3.1. In vitro experiments on guinea-pig isolated ileum

Guinea pig ileum was chosen because it represents a suitable model for studying intestinal contractile response.

MED15 demonstrated a dose-related inhibitory effect on ileum stimulated with various agonists. It antagonized, at all the tested doses, the cumulative curve induced by histamine (from 10^{-9} to 10^{-7} M), with significant effect from 12.5 μM (the lowest dosage used) to 50 μM; also after stimulation with a single dose of histamine, a dose–effect relationship was in evidence (Fig. 1A and B). In single-dose experiments, the agonist dose was selected to produce a contractile effect capable of determining 80–90% of maximal effects, as found in preliminary experiments.

Fig. 2A and B shows the effect of MED15 on acetylcholine-stimulated guinea-pig ileum: it is clear that MED15 dose-dependently inhibits contractions due to single and cumulative doses of acetylcholine. This agonist is known to increase peristalsis and contraction breadth of the gastroenteric tract (Fox-Orenstein and Grider, 1996) and MED15 at the tested doses consistently shows a statistically significant inhibitory response.

The effect of MED15 on guinea-pig isolated ileum following stimulation with serotonin is shown in Fig. 3. 5-Hydroxytryptamine controls motility in the gastroenteric tract: MED15 dose-dependently inhibits contractions induced by this agonist, showing total inhibition at a dose of 100 μM, and a significant antispasmodic effect at the other concentrations up to 6.25 μM, where 37% inhibition of contractile response was still evident.

A similar inhibitory effect was observed using bradykinin (Table 1). When the ileum segments were stimulated with this agonist, MED15 showed a dose-related inhibition from 100 to 12.5 μM (the lowest tested dose) and compared favourably with all of the reference compounds. MED5 and tolmetin, its active metabolites, showed a different type of behaviour: tolmetin did not influence bradikinin-induced contractions, with low effect only at the highest doses; MED5 determined a 40.6 ± 5.7% and 48.1 ± 7.1% inhibition at 100 and 200 μM, respectively.

Indomethacin and nabumetone at 100 μM showed a maximal effect much lower than the corresponding dose of MED15 (58.3 and 71.5%, respectively, vs. 100% of MED15).

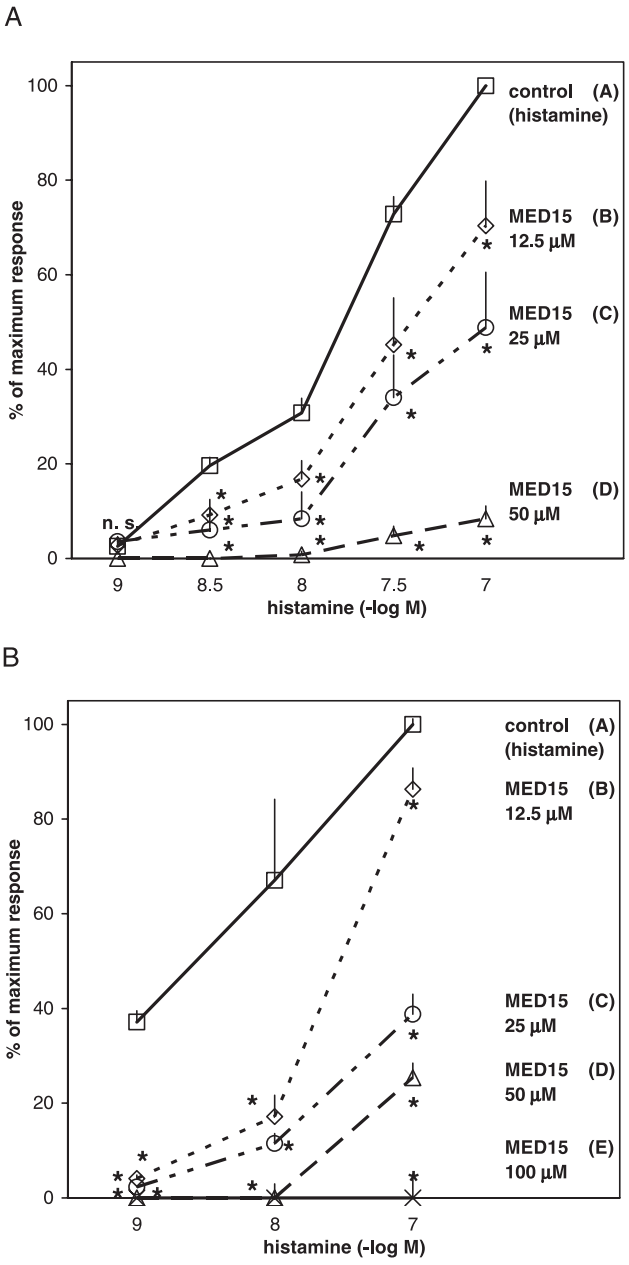

Fig. 1. In vitro effect of MED15 on response of guinea-pig ileum to histamine. MED15 was tested at doses of 12.5, 25, 50 and 100 μM. Results are expressed as percentage of the maximum response induced by the agonist and values are mean ± S.E. of three experiments in duplicate. Statistical analysis: one-way analysis of variance followed by Student–Newman–Keuls test. *Statistical significance vs. histamine control ($P<0.05$). (A) Cumulative curve: histamine from 10^{-9} to 10^{-7} M. Significant differences between groups: at histamine from 10^{-8} to 10^{-7} M B vs. D; at histamine 10^{-7} M C vs. D. (B) Single dose: histamine 10^{-9}, 10^{-8} and 10^{-7} M. Significant differences between groups: at histamine 10^{-8} M B vs. C, B vs. D, B vs. E, C vs. E.

3.2. Effect of MED15 on indomethacin-induced intestinal inflammation in the rat

Acute administration of indomethacin to rodents produces gastrointestinal inflammation (Brodie et al., 1970) and

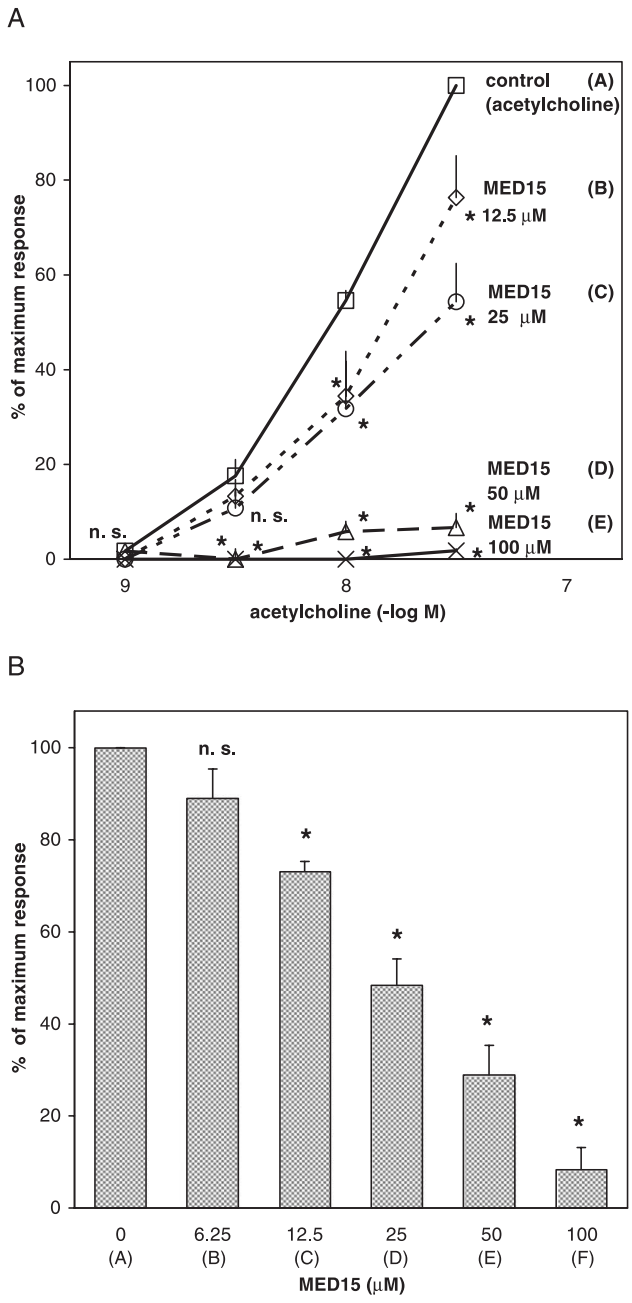

Fig. 2. In vitro effect of MED15 on response of guinea-pig ileum to acetylcholine. MED15 was tested at doses of 6.25, 12.5, 25, 50 and 100 μM. Results are expressed as percentage of the maximum response induced by the agonist and values are mean ± S.E. of three experiments in duplicate. Statistical analysis: one-way analysis of variance followed by Student–Newman–Keuls test. *Statistical significance vs. acetylcholine control ($P<0.05$). (A) Cumulative curve: acetylcholine from 10^{-9} to 5×10^{-8} M. Significant differences between groups: at acetylcholine 10^{-8} M B vs. D, B vs. E, C vs. D, C vs. E; at acetylcholine 5×10^{-8} M B vs. C, B vs. D; B vs. E, C vs. D, C vs. E. (B) Single dose: acetylcholine at 10^{-7} M. All comparisons between groups were significant.

repeated injections of this NSAID produce extensive chronic inflammation lasting in the active form for at least 2 weeks. This model of inflammatory bowel disease is reported to mimic human gastrointestinal inflammation

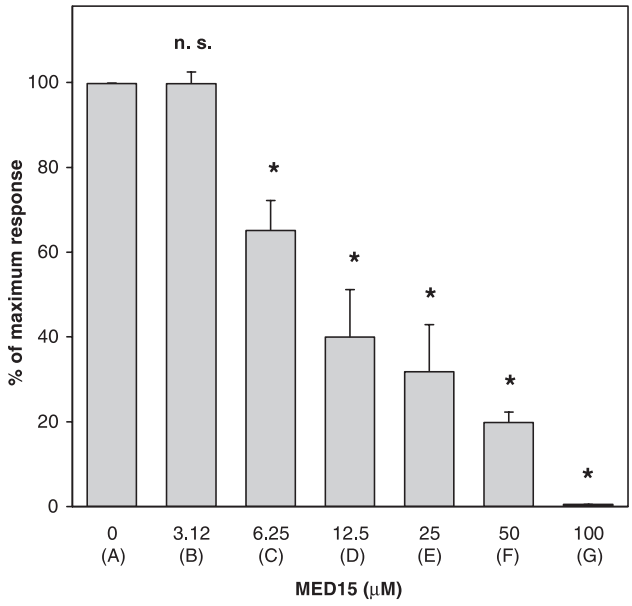

Fig. 3. In vitro effect of MED15 on response of guinea-pig ileum to a single dose of 5-hydroxytryptamine (10^{-7} M). MED15 was tested at doses of 3.12, 6.25, 12.5, 25, 50 and 100 μM. Results are expressed as percentage of the maximum response induced by the agonist and values are mean ± S.E. of three experiments in duplicate. Statistical analysis: one-way analysis of variance followed by Student–Newman–Keuls test. *Statistical significance vs. 5-hydroxytryptamine control ($P<0.05$). D vs. E: n.s.; D vs. F: n.s.; E vs. F: n.s.

(Kucharzik et al., 2000). In the authors' experiments, all parameters evaluated in this model showed anti-inflammatory effect of MED15 superior to that of 5-aminosalicylic acid and sulfasalazine. Administration of indomethacin induced intestinal inflammation characterised, compared to vehicle-treated controls, by significant development of

Table 1
Effect of MED15 and various NSAIDs on bradykinin-stimulated guinea-pig isolated ileum

Drug	Concentration (μM)	Percentage inhibition of maximal response
MED15	100.0	100.0 ± 0.2
	50.0	91.1 ± 8.7
	25.0	51.5 ± 4.9
	12.5	31.8 ± 3.9
Indomethacin	200.0	73.0 ± 7.8
	100.0	58.3 ± 6.0
	50.0	31.4 ± 3.8
MED5	200.0	48.1 ± 7.1
	100.0	40.6 ± 5.7
	50.0	18.2 ± 6.3
Tolmetin	200.0	18.2 ± 2.7
	150.0	15.4 ± 5.1
	100.0	0
Nabumetone	200.0	78.3 ± 8.6
	100.0	71.5 ± 6.9
	50.0	28.0 ± 4.6

Results are expressed as mean ± S.E. of three experiments in duplicate. Bradykinin dose: 10^{-7} M.

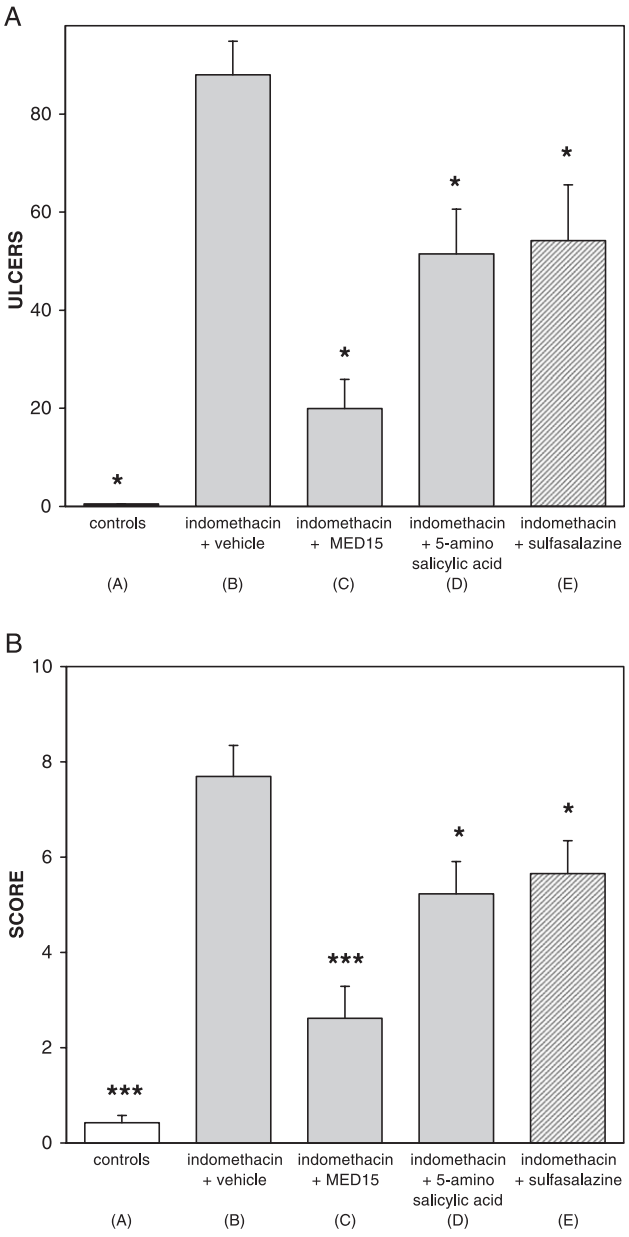

Fig. 4. In vivo effect of MED15 on indomethacin-induced small bowel inflammation. Rats were treated subcutaneously with indomethacin (7.5 mg/kg) at days 0 and 1; MED15, 5-aminosalicylic acid and sulfasalazine (100 mg/kg p.o.) were given at days −1, 0, 1, 2 and 5. Data are expressed as mean ± S.E. values from nine rats. Statistical analysis for ulcers: one-way analysis of variance followed by Student–Newman–Keuls test; statistical analysis for score: Mann–Whitney U test. (A) *Statistical significance ($P<0.05$) calculated vs. B. Other significant differences between groups: C vs. D, C vs. E. (B) Statistical significance calculated vs. B: *$P<0.05$; **$P<0.01$; ***$P<0.001$. Other significant differences between groups: A vs. C: **; A vs. D and A vs. E: ***; C vs. D: *; C vs. E: **.

ulcers (88 ± 6.82 and 0, respectively) and an increase in score index (7.75 ± 0.49 and 0.33 ± 0.16, respectively).

A statistically significant drop (77.3%) in the number of ulcers was observed in the MED15-treated group compared to the group receiving indomethacin alone, with a 41.5% reduction following 5-aminosalicylic acid administration, and of 38.4% following sulfasalazine (Fig. 4A). Animals treated with indomethacin and MED15 are not significantly different from vehicle-treated controls, but show a significant difference compared to the 5-aminosalicylic acid and sulfasalazine-treated ones.

The scores of the MED15-treated animals also showed significant drops compared to the indomethacin-treated controls (2.38 ± 0.53 and 7.75 ± 0.49, respectively, $P \leq 0.001$). The 5-aminosalicylic acid and sulfasalazine-treated animals had scores of 5.31 ± 0.85 ($P \leq 0.05$) and 5.44 ± 0.74 ($P \leq 0.05$) (Fig. 4B).

Results reported in Table 2 show intestinal damage-related variation in the length of small intestine, body weight and body weight/bowel length index.

Body weights in all groups of animals treated with indomethacin are similar and are significantly different compared to controls. Bowel length (expressed in centimeters) in the indomethacin-treated group is 54.4 ± 2.03 with a significant reduction compared to the vehicle-treated group (90.0 ± 3.53); after treatment with MED15, 5-aminosalicylic acid and sulfasalazine, bowel length is 80.1 ± 4.45, 71.4 ± 4.87 and 75.6 ± 7.94 cm, respectively, with a statistical significance vs. indomethacin.

MED15-treated animals show a body weight/bowel length index drop compared to indomethacin-treated rats, demonstrating the regression of bowel inflammation. Also, animals treated with 5-aminosalicylic acid and sulfasalazine show similar behaviour in this parameter, although the drop is less evident here.

3.3. Myeloperoxidase determination

Myeloperoxidase (known to be a marker of polymorph infiltration), in the indomethacin-treated group shows an

Table 2
Effect of MED15 and reference compounds on changes in body weight, bowel length and body weight/bowel length index in indomethacin-induced bowel inflammation

Groups	Body weight (g)	Bowel length (cm)	*w/l* index
(A) $NaHCO_3$ 5% + CM-Cellulose 1%	299.3 ± 10.9[a]	90.0 ± 3.53[a]	3.28 ± 0.1[a]
(B) Indomethacin + CM-Cellulose 1%	247.2 ± 20.9	54.4 ± 2.03	4.73 ± 0.51
(C) Indomethacin + MED15	251.4 ± 9.8 n.s.	80.1 ± 4.45[a]	3.22 ± 0.24[a]
(D) Indomethacin + 5-aminosalicylic acid	249.5 ± 4.6 n.s.	71.4 ± 4.87[a]	3.66 ± 0.3[a]
(E) Indomethacin + Sulfasalazine	249.1 ± 4.2 n.s.	75.6 ± 7.94[a]	3.51 ± 0.24[a]

Values are mean ± S.E. of nine animals/group.
Statistical evaluation shown in the table was calculated against group B (one-way analysis of variance followed by Student–Newman–Keuls test).
[a] Statistical significance, $P<0.05$. Other significant between-group differences were found only for body weight: A vs. C; A vs. D; A vs. E.

increase of 91.7% compared to vehicle-treated controls (3.0 ± 0.18 and 1.57 ± 0.14 U/mg wet tissue, respectively). After treatment with indomethacin and MED15, myeloperoxidase activity falls to 2.21 ± 0.09 showing a significant reduction compared to indomethacin; administration of 5-aminosalicylic acid and sulfasalazine determines the following values in myeloperoxidase activity: 2.41 ± 0.34 (n.s.) and 2.07 ± 0.29 ($P \leq 0.05$), respectively (Fig. 5).

3.4. L-NAME effect in indomethacin-induced intestinal inflammation

In these experiments, selected animals were of lower weight compared to those used in the other experiments, to make them hyporesponsive to indomethacin treatment, thus permitting better evaluation of L-NAME-induced exacerbating effect (no published data). Fig. 6 shows that L-NAME amplifies indomethacin-induced intestinal damage: number of ulcers (indomethacin 9.61 ± 6.5; indomethacin + L-NAME 53 ± 9.1) and score (indomethacin 3.94 ± 0.52; indomethacin + L-NAME 8.61 ± 0.65) increase, indicating the importance of NO in maintaining integrity of the intestinal mucosa.

In this experimental model, MED15 shows similar percentages of damage inhibition both in the animals treated with indomethacin alone and in those treated with indomethacin + L-NAME (-86.1% and -82.3%, respectively, as number of ulcers and -66.2% and -61.3%, respectively, for the score), maintaining its level of significant protective activity even when NO synthase is inhibited. It must be marked that there is no statistical difference between MED15-treated animals vs. vehicle-treated controls in number of ulcers.

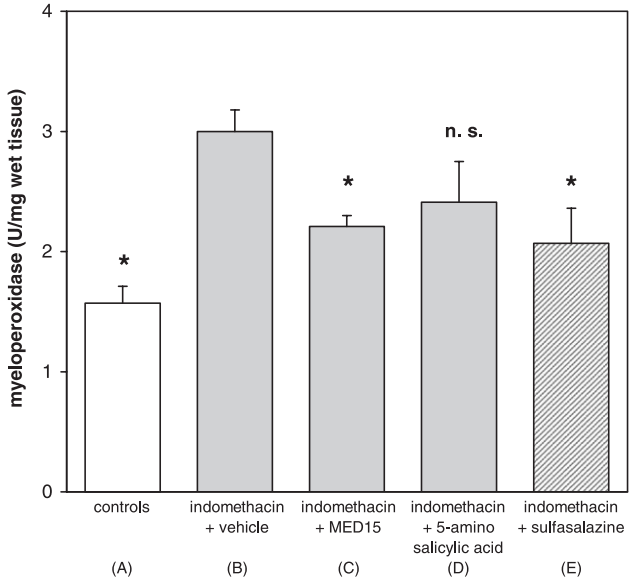

Fig. 5. Effect of MED15 on myeloperoxidase activity in indomethacin-induced small bowel inflammation. Rats were treated subcutaneously with indomethacin (7.5 mg/kg) at days 0 and 1; MED15, 5-aminosalicylic acid and sulfasalazine (100 mg/kg p.o.) were given at days -1, 0, 1, 2 and 5. Data are expressed as mean ± S.E. values from eight rats. Statistical analysis: one-way analysis of variance followed by Student–Newman–Keuls test. *Statistical significant ($P < 0.05$) vs. B. No significant difference between groups other than that reported in figure was obtained.

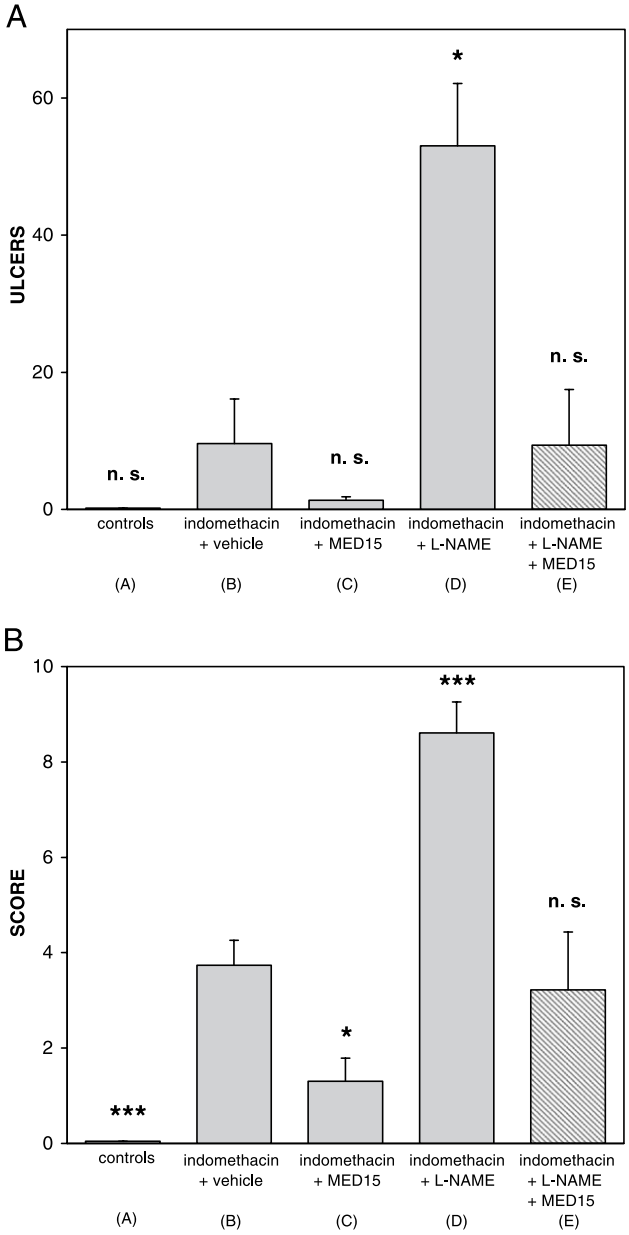

Fig. 6. In vivo effect of MED15 on indomethacin-induced small bowel inflammation in L-NAME-treated rats. L-NAME (5 mg/kg s.c.) and MED15 (100 mg/kg p.o.) were administered simultaneously at days -1, 0, 1, 2 and 5. Indomethacin (7.5 mg/kg s.c.) was given at days 0 and 1. Ulcers number (A) and score values (B) were determined. Data are expressed as mean ± S.E. values from nine rats. Statistical analysis for ulcers: one-way analysis of variance followed by Student–Newman–Keuls test; statistical analysis for score: Mann–Whitney U test. (A) *Statistical significance ($P < 0.05$). D vs. E: *. (B) Statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. A vs. C: n.s.; D vs. E: **.

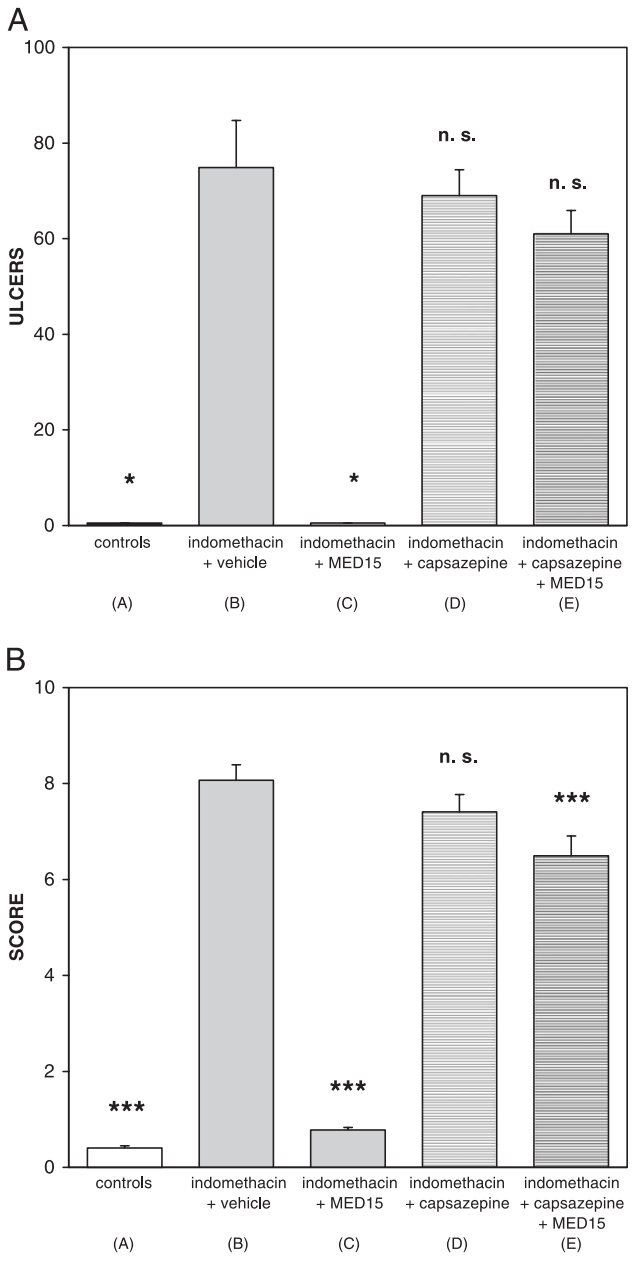

Fig. 7. Effect of capsazepine and MED15 on indomethacin-induced small bowel inflammation. Rats were treated subcutaneously with indomethacin (7.5 mg/kg) at days 0 and 1; capsazepine (77 μmol/kg s.c.) and MED15 (100 mg/kg p.o.) were administered simultaneously at days − 1, 0, 1, 2 and 5. Ulcers number (A) and score values (B) were determined. Data are expressed as mean ± S.E. values from nine rats. Statistical analysis for ulcers: one-way analysis of variance followed by Student–Newman–Keuls test; statistical analysis for score: Mann–Whitney *U* test. (A) *Statistical significance ($P<0.05$). A vs. D*, A vs. E*, C vs. D*, C vs. E*. (B) Statistical significance: ***$P<0.001$. No significant difference between groups other than that reported in figure was obtained.

3.5. Capsazepine effect in indomethacin-induced intestinal inflammation

In this model, it was manifest that capsazepine was ineffective toward the intestinal damage produced by indomethacin: the ulcer number and score values are not statistically different in these two groups.

In the presence of capsazepine, MED15 loses its protective effect, as demonstrated by the number of ulcers (indomethacin + capsazepine: 69.0 ± 5.4.; indomethacin + capsazepine + MED15: 61 ± 4.9 n.s.) and by the score values (indomethacin + capsazepine: 7.41 ± 0.36 and indomethacin + capsazepine + MED15: 6.49 ± 0.42 n.s.).

In this experiment, MED15, administered in absence of capsazepine, confirms its protective effect on intestinal inflammation, producing a 90.3% drop in score value and a 100% drop in number of ulcers (Fig. 7A and B).

3.6. Malondialdehyde determination in acetic acid-induced colitis

In acetic acid-induced colitis, malondialdehyde levels, representing the product of oxidative degradation of unsaturated fatty acids, permitted a good evaluation of the inflammatory condition. The following values were obtained: vehicle-treated controls 622 ± 51.2 nmol/g, acetic acid 810 ± 40.7 nmol/g, acetic acid + MED15 589 ± 31.8 nmol/g and acetic acid + 5-aminosalicylic acid 723 ± 58.9 nmol/g. In this model, MED15 demonstrated more pronounced antioxidative effects than 5-aminosalicylic acid (MED15 − 27.2%, $P<0.05$; 5-aminosalicylic acid − 10.7% n.s.) (Fig. 8).

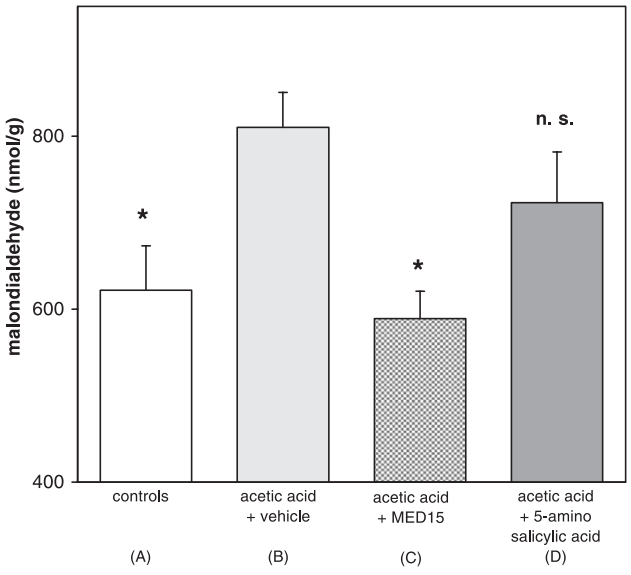

Fig. 8. Effect of MED15 on malondialdehyde levels in acetic acid-induced colitis in the rat. MED15 (100 mg/kg p.o.) and 5-aminosalicylic acid (400 mg/kg p.o.) were administered 48, 24 and 2 h before the instillation of 3% acetic acid (1.5 ml/rat) into the lumen of the distal colon. Malondialdehyde levels were determined 24 h after induction of colitis. Data are expressed as mean ± S.E. values from six rats. Statistical analysis: one-way analysis of variance followed by Student–Newman–Keuls test. *Statistical significance ($P<0.05$). No significant difference between groups other than that reported in figure was obtained.

4. Discussion

It is known that capsaicin receptor stimulation, besides producing gastroprotective effects (Holzer and Sametz, 1986; Holzer and Lippe, 1988;), also produces pharmacological effects on the intestine (Bartho et al., 1987; Evangelista and Meli, 1989; Maggi et al., 1989). Gastric capsaicin receptor stimulation, through the vanillic moiety of the MED15 molecule, elicited evidence of a protective effect on gastric mucosa in the rat (Whittle et al., 1995; Tubaro et al., 2000) and of misoprostol-like activity in human volunteers (Riezzo et al., 2001); these data represent the rationale of the exceptional gastric tolerance of the drug along with the good anti-inflammatory efficacy found in clinical practice (Marcolongo et al., 1999).

Due to the strong and persistent presence of the molecule as such in the walls throughout the gastroenteric tract and to the known presence of capsaicin receptors in the gut (Bartho et al., 1987), the authors investigated both the antispasmodic effects of the drug on the isolated intestine, stimulated with a variety of agonists, and the protective effect on in vivo models of intestinal inflammation. Capsaicin receptors, in fact, play a complex role on the bowel physiology and capsaicin-sensitive fibres were demonstrated to exercise a protective influence on experimentally induced colitis in the rat (Stein et al., 1986; McCafferty et al., 1997; Szepes et al., 1997). Additionally, stimulation of capsaicin receptors on intestinal mucosa determines a release of neuropeptides (Evangelista et al., 1987) and NO (Roberts et al., 2001). MED15's intestinal protection mechanism appears to be unrelated to increased production of NO, because the MED15-related reduction of indomethacin-induced ulcers occurs also in the presence of L-NAME (a NO synthase-specific inhibitor): MED15 effect on intestinal damage was attributed to capsaicin receptor stimulation and this was verified in an in vivo model of indomethacin-induced bowel damage. In this experiment, the MED15 protective effect was lost in the presence of capsazepine, a specific capsaicin receptor inhibitor: this demonstrates that the protective effect of MED15 is due to capsaicin receptor stimulation and the probable, consequent release of neuropeptides.

The results of the present study appear to point toward possible use of MED15 in various inflammatory conditions of the intestine, because it has demonstrated high efficacy in in vivo models.

Moreover, the antispasmodic activity of MED15 shown in in vitro studies appears to be attributable to the presence of a vanillic moiety in the molecule. In fact, the inhibitory effect on histamine-induced contractions was not unexpected, due to the opposing effects of histamine and capsaicin in non-adrenergic, non-cholinergic sensory nerve stimulation (Matusak and Bauer, 1988). The impact of MED15 on the isolated ileum stimulated with 5-hydroxytryptamine, known to control gastrointestinal motility (Gonella, 1981), may be attributable to the efficacy of capsaicin in inhibiting the effects of 5-hydroxytryptamine (Virus and Gebhart, 1979; Rolfe and Levin, 1998). Further demonstration of the correlation between the antispasmodic effects of MED15 and capsaicin receptors are the results obtained in the isolated ileum stimulated with bradykinin, a known agonist of both inflammation and inflammatory pain. Bradykinin stimulates electrolyte secretion of the intestinal wall and short circuit currents with involvement of arachidonic acid and its metabolites (Musch et al., 1983); consequently, all NSAIDs, acting on arachidonic acid metabolism, are known to partially block the effects of bradykinin. The more pronounced anti-spasmodic effect of MED15 compared to that of the other NSAIDs may be explained by the inhibitory effect of capsaicin, which down-regulates the bradykinin-induced reflex excitation of sympathetic activity of intestinal afferent nerves (Stein et al., 1986). Further evidence that MED15's anti-bradykinin effect is due to the capsaicin radical found in its molecule is the scarce anti-bradykinin effect of the parent molecule tolmetin at the tested doses.

The inhibitory effect on ileum contraction, following stimulation with various agonists, and the antimuscarinic effect of MED15, similar to belladonna alkaloids, metanteline and pirenzepine, verified using acetylcholine (which increases peristalsis and contraction breadth of the gastroenteric tract) (Fox-Orenstein and Grider, 1996), support possible use of amtolmetin guacyl as an antispasmodic agent in bowel disease.

In our studies, animal models were diversified in the attempt to mimic human intestinal disease; in particular, indomethacin was used to induce acute ileitis and acetic acid to produce specific colon inflammation. NSAID enteropathy is probably one of the most interesting animal models for Crohn's disease (Yamada et al., 1993) because it shares the same early modification observed in the human disease, and that is, increased intestinal permeability, followed by inflammatory lesions (Louis and Belaich, 1994). All parameters examined in our experimental model of indomethacin-induced intestinal inflammation highlighted the favourable impact of MED15 on damaged intestinal walls, compared with 5-aminosalicylic acid and sulfasalazine, reference drugs currently used for the treatment of colitis. In the reported study, MED15 also caused a significant reduction of intestinal wall myeloperoxidase content, a marker of injury caused by neutrophil infiltration leading to disruption of colon epithelial barrier function (Ginzberg et al., 2001).

Besides indomethacin-induced ileitis miming of human chronic intestinal inflammation (Kucharzik et al., 2000), acetic acid produces colitis similar to human inflammatory bowel disease as well (Sharon and Stenson, 1985). In the current model, malondialdehyde was evaluated as a biochemical marker of oxidative stress in inflammation (Cuzzocrea et al., 2001); MED15 shrank malondialdehyde levels better than 5-aminosalicylic acid.

However, animal models used in pharmacology for the induction of colitis or ileitis only partially mimic human pathology. Acute colitis, induced in the rat, represents a

suitable model because of its similarity to human colitis inasfar as neutrophil infiltration and arachidonic acid metabolism is concerned; nevertheless, it differs in the lack of relapse episodes characteristic of chronic inflammation, and of autoimmune component generation typical of human disease. Evaluation of the results obtained probably underestimates the potential efficacy of MED15 in human disease, an indication being the modest or null attenuation of inflammatory states obtained in animals by reference drugs of common clinical use and proven efficacy in the treatment of human ulcerative colitis, such as 5-aminosalicylic acid and sulfasalazine.

In conclusion, notwithstanding the paradox of its NSAID nature, it appears rationally possible to propose the use of MED15 in the complex field of human bowel disease, on the basis of the encouraging and molecule-related results obtained in pharmacological models.

Additionally, the exceptionally good gastroenteric tolerance found in clinical practice in the course of several years of daily MED15 administration, at times of several months' duration, assures safety and suitability in long-term treatment.

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