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DIRECCIÓN DE NUEVAS CREACIONES**

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Abogado
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ASUNTO	Radicación	10 002 341
	Trámite	11
	Evento	378
	Actuación	519
	Oficio	
	Folios	11

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Patente de Invención	☒	Patente de Modelo de Utilidad
Vía Nacional		
Vía PCT (Fase Nacional)	☒	Capítulo I ☒ Capítulo II
No. Solicitud Internacional	PCT/EP2008/059296	
Fecha de Presentación Internacional	16/Julio/2008	

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 38 a 90	12/Enero/2010
Reivindicaciones:	Folios 91 a 95	12/Enero/2010
Dibujos:	Folios 97 a 99	12/Enero/2010
Prioridad:	EP 07112679.1	18/Julio/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Combinaciones sinérgicas de antagonistas de VR-1 e inhibidores de COX-2".

El problema planteado en esta solicitud consiste en la necesidad suministrar mejores agentes terapéuticos antagonistas del receptor de capsaicina VR-1.

Para resolver este problema técnico la solicitud en estudio proporciona una combinación sinérgica de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo, o una sal o solvato farmacéuticamente aceptable de

cualquiera o ambos compuestos.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K45/06//31/4418//31/517, A61P29/00.

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de la reivindicación 7 no es patentable, de acuerdo con el Art 14.

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara cuando emplea el término “sinérgica”, lo cual hace referencia a un resultado a alcanzar, porque no define a la combinación, la invención no queda definida por el resultado a alcanzar, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere eliminar el término del pliego reivindicatorio y reemplazarlo por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese efecto.

Las reivindicaciones 1, 2 y 4 no son claras cuando emplean los términos “antagonista del receptor vainilloide VR-1”, “antiinflamatorio no-esteroideo”, “inhibidor de COX-2”, “compuesto de fórmula (I)”, “compuestos de fórmula (II)”, porque se definen los componentes de la combinación por su función o por su estructura química común, pero no por un compuesto como tal, lo que hace que se obtengan un número indefinido de combinaciones que no encuentran soporte en la descripción. Se sugiere al solicitante restringir el alcance de la reivindicación a componentes específicos debidamente soportados en la descripción.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad 18 de julio de 2007 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D1	WO02076946	Pyridine derivatives	03/Octubre/2002
D2	WO2005120510	Quinazolinone derivatives useful as vanilloid antagonists	22/Diciembre/2005
D3	WO2004056394	Combinations of a vanilloid antagonist and an NSAID for the treatment of pain	08/Julio/2004
D4	WO2006124753	Treatment of VR1-antagonist-induced increase in body temperature with an antipyretic agent	23/Noviembre/2006
D5	WO2007074916	3,4-dihydrobenzoxazine compound and inhibitor of vanilloid receptor type 1 (VR1) activity	05/Julio/2007
D6	US2007032500	Therapeutic agents useful for treating pain	08/Febrero/2007
D7	Artículo ¹	Impact on the bowel of amtolmetin guacyl, a new gastroprotective non-steroidal anti-inflammatory drug	23/Abril/2003

¹ Tubaro, et al; Impact on the bowel of amtolmetin guacyl, a new gastroprotective non-steroidal anti-inflammatory drug; European Journal of Pharmacology; Vol. 467, 2003, pg. 173-183, folios 125-130.

D1

http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=02076946A2&KC=A2&FT=D&ND=&date=20021003&DB=&locale=en_EP, divulga (reivindicaciones 1, 2, 12, pg. 8) una combinación de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo, y composiciones farmacéuticas que la contienen.

D2

http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2005120510A1&KC=A1&FT=D&ND=&date=20051222&DB=&locale=en_EP, divulga (pg. 13 – 14, reivindicaciones) una combinación de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo, y composiciones farmacéuticas que la contienen.

D3

http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2004056394A1&KC=A1&FT=D&ND=&date=20040708&DB=&locale=en_EP, divulga (todo el documento) una combinación de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo, y composiciones farmacéuticas que la contienen.

D4

http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2006124753A2&KC=A2&FT=D&ND=&date=20061123&DB=&locale=en_EP, (reivindicaciones) divulga una combinación de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo, y composiciones farmacéuticas que la contienen.

D5

http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2007074916A1&KC=A1&FT=D&ND=&date=20070705&DB=&locale=en_EP, divulga (reivindicaciones 1, 2, 15; pár. 0136- 0151) una combinación de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo, y composiciones farmacéuticas que la contienen.

D6

http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=2007032500A1&KC=A1&FT=D&ND=&date=20070208&DB=&locale=en_EP, divulga (pár. 0712-0721) una combinación de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo, y composiciones farmacéuticas que la contienen.

D7 (folios 125 – 130) divulga (pg. 181, fl 129) una combinación de un capsaicina como antagonista del receptor vainilloide VR-1, y MED15, un fármaco anti-inflamatorio no esteroideo.

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en una combinación sinérgica de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo, o una sal o solvato farmacéuticamente aceptable de cualquiera o ambos compuestos.

Comparación de las características técnicas esenciales, con las del Estado de la Técnica:

Características esenciales	D1	D2	D3	D4
Combinación de:	Combinación de (reiv. 12):	Combinación de (pg. 13 – 14)	Combinación de (abstract)	Combinación de (reivindicaciones)
un antagonista del receptor vainilloide VR-1, su sal o solvato farmacéuticamente aceptable y	un antagonista del receptor vainilloide VR-1 (reivindicaciones 1 y 2)	un antagonista del receptor vainilloide VR-1 (reivindicaciones 1 y 4, ejemplos)	un antagonista del receptor vainilloide VR-1 (abstract)	un antagonista del receptor vainilloide VR-1 (reivindicaciones)
un fármaco anti-inflamatorio no esteroideo, su sal o solvato farmacéuticamente aceptable	Un inhibidor de COX-2 (pg. 8)	Un inhibidor de COX-2 (pg. 13, pár. 3)	Un antiinflamatorio no esteroide (abstract)	Un antipirético (como un AINES) (reivindicaciones)

Comparación de las características técnicas esenciales, con las del Estado de la Técnica:

Características esenciales	D5	D6	D7
Combinación de:	Combinación de (reivindicación 15):	Combinación de (pár. 0712-0713)	Combinación de (abstract)
un antagonista del receptor vainilloide VR-1, su sal o solvato farmacéuticamente aceptable y	un antagonista del receptor vainilloide VR-1 (reivindicaciones 1, 2 y 15)	un antagonista del receptor vainilloide VR-1 (pár. 0713)	Capsaicina (pg. 181, col. Izquierda, fl 129)
un fármaco anti-inflamatorio no esteroideo, su sal o solvato farmacéuticamente aceptable	Un antiinflamatorio no esteroide (reivindicación 15, pár. 146)	Un AINES, o un inhibidor de COX-2 (pár. 0717)	MED15 (un antiinflamatorio no esteroide) (abstract)

Las características esenciales de la combinación de la reivindicación 1 habían sido divulgadas previamente en D1, D2, D3, D4, D5, D6 y D7, los cuales enseñan la combinación de un antagonista del receptor vainilloide VR-1, y un fármaco anti-inflamatorio no esteroideo (D1: reivindicaciones 1, 2 y 12; D2: págs. 13 y 14, reivindicaciones; D3: abstract; D4: reivindicaciones; D5: reivindicaciones 1, 2 y 15; D6: pár. 0712-0717; D7: pg. 181).

En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en los documentos D1, D2, D3, D4, D5, D6 o D7.

Además, D1 y D2 enseñan que el antagonista del receptor vainilloide VR-1 puede ser 7-terbutil-6-(4-cloro-fenil)-2-tioxo-2,3-dihidro-1-H-pirido-[2,3-d]-pirimidin-4-ona o 4-(7-hidroxi-2-isopropil-4-oxo-4H-quinazolin-3-il)-benzonitrilo (D1: pg. 12, ejemplo 2; D2: ejemplo 29).

Asimismo, D1, D2, D3, D5 y D6 enseñan que el antiinflamatorio no-esteroideo corresponde a un inhibidor de COX-2 seleccionado de celcoxib, rofecoxib, parecoxib, 2-(4-etoxi-fenil)-3-(4-metan-sulfonil-fenil)pirazolo-[1,5-b]-piridazina, entre otros (D1: pg. 8, pár. 4; D2:pg. 13, pár. 3; D3: pg. 38; D5: pár. 0146; D6: pár. 0721).

Las reivindicaciones dependientes 1 - 7 no mencionan alguna característica que haga nueva a la combinación de la reivindicación 1, por lo cual se considera que no son nuevas.

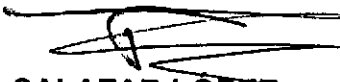
8. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO02076946	1 - 7	Novedad
D2	WO2005120510	1 - 7	Novedad
D3	WO2004056394	1 - 4 y 6 - 7	Novedad
D4	WO2006124753	1, 4 y 6 - 7	Novedad
D5	WO2007074916	1 - 3 y 6 - 7	Novedad
D6	US2007032500	1 - 3 y 6 - 7	Novedad
D7	WO2006007851	1 y 6	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


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Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 11 SET. 2012

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Impact on the bowel of amlodipine guanyl, a new gastroprotective non-steroidal anti-inflammatory drug

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Abstract

Amlodipine guanyl (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 antipainful 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 sulphasalazine, 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 enteroprotective 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 capsaizine, 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.

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Keywords: Amlodipine guanyl; Isomeric; Inflammation; Neuroepithelium; (Rat)

1. Introduction

Amlodipine guanyl (2-methoxyphenyl-1-methyl-5-p-methyl benzoyl-pyrrol-2-acetamide 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; Petuzzi et al., 1990a,b; Simon et al., 1998; Bianchi Porro et al., 1999; Lanza, 1999; Marcolongo et al., 1999; Montone 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 indo-

methacin-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 Gault, 1995). Capsaicin-like behaviour of MED15 was demonstrated in several studies using either capsaizine, 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 mitoprostol-like effect of MED15 was demonstrated using electrogastragraphy (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 gastrointestinal 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 the 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 writhings 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 dihydrochloride, 5-hydroxytryptamine hydrochloride dissolved in Tyrode's solution: 0.1 M-acetate, 10 mM potassium phosphate buffer, acetic acid, hexacetyltrimethylammonium bromide, hydrogen peroxide, sodium acetate trihydrate, dimethylsulfoxide (DMSO), potassium sulphate, CM-Celulose and 1,1,3,3-tetraethoxypropane dissolved in water, 2-thiobarbituric acid in 2 M sodium acetate buffer, butylhydroxytoluene in absolute ethanol, *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME) in saline, capsaizine in 50% ethanol, indomethacin dissolved in NaHCO₃, 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

Amlodipine guanyl (MED15) (Medason Ricerca, Italy), 5-aminosalicylic acid and sulphasalazine (Sigma) dissolved in CM-Celulose 1% for *in vivo* studies.

MED15, *N*-(1-methyl-5-(4-methylbenzoyl)-1H-pyrrol-2-yl)-glycine (MEDS) (Medason Ricerca, Italy), nabum-

etone 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 ileocecal 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; CaCl₂ 1.6; MgCl₂ 1.1; NaH₂PO₄ 0.5; NaHCO₃ 7.7; glucose 5.6) gassed with 95% O₂:5% CO₂ and maintained at 37 °C. The tissue was loaded with 1.0 g and contractions were detected isometrically by a force transducer (model, 7003, Basile, Italy) equipped with a recording dynamometer (model, 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, MEDS, 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 NaHCO₃, 5% (2.5 ml/kg) for 2 consecutive days (days 0 and 1). MED15, 5-aminosalicylic acid and sulphasalazine 100 mg/kg, were administered orally in CM-Celulose 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 (standardized 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 (Whatman 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 (Yanada 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 ulcerations, 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. *c-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 *c-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 $\mu\text{mol/kg}$ 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% hexadecyltrimethylammonium bromide in 50 mM

potassium phosphate buffer (pH 6.0) to give a 30 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 fisted 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 7–48, –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-tetrahydroxypropane 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% butyldiethylamine 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 Petkin-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 Bondapak C18 (250 $\times$ 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 $\pm$ 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 Ginder, 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. MED15 and tolmetin, its active metabolites, showed a different type of behaviour: tolmetin did not influence bradykinin-induced contractions, with low effect only at the highest doses; MED15 determined a $40.6 \pm 5.7\%$ and $48.1 \pm 7.1\%$ 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).

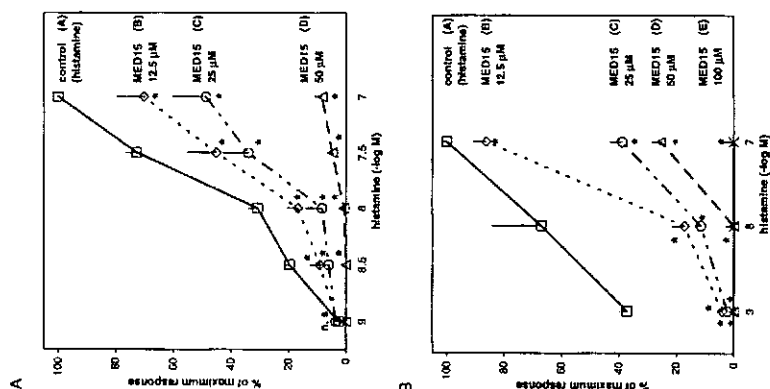


Fig. 1. *In vitro* effect of MED15 on response of guinea-pig ileum to histamine. MED15 was tested at doses of 12.5, 25, 50 and 100 μM . Results are expressed as percentage of the maximum response induced by the agonist and values are mean $\pm$ S.E. of three experiments in duplicate. Statistical analysis: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

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 ± 0.5 ; indomethacin + L-NAME 33 ± 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

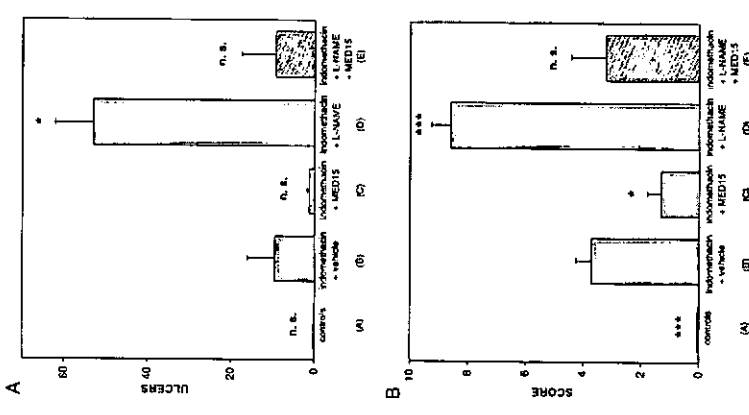


Fig. 6. In vivo effect of MED15 on L-NAME-induced small bowel inflammation in L-NAME-treated rats. L-NAME (5 mg/kg s.c.) and MED15 (100 mg/kg p.o.) were administered simultaneously at days $-1, 0, 1, 2$ and 5. Indomethacin (7.5 mg/kg s.c.) was given at days 0 and 1. Ulcers number (A) and score values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

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.

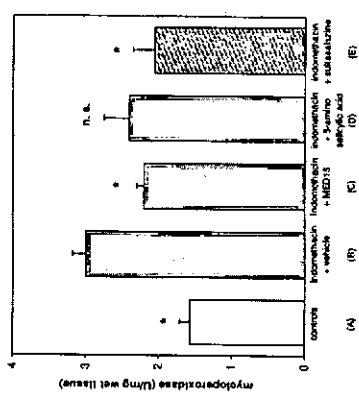


Fig. 5. Effect of MED15 on myeloperoxidase activity in indomethacin-induced small bowel inflammation. Rats were treated subcutaneously with indomethacin (7.5 mg/kg) at days 0 and 1; MED15, 5-aminosalicylic acid and sulfasalazine (100 mg/kg p.o.) were given at days $-1, 0, 1, 2$ and 5. Data are expressed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

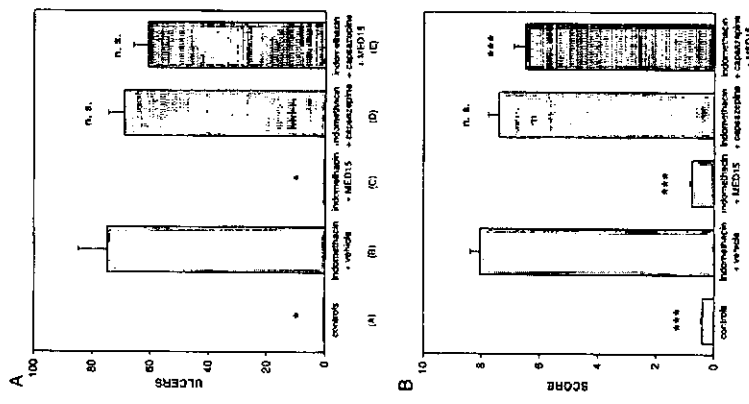


Fig. 7. Effect of capsaicin and MED15 on indomethacin-induced small bowel inflammation. Rats were treated subcutaneously with indomethacin (7.5 mg/kg) at days 0 and 1; capsaicin (77 μ mol/kg s.c.) and MED15 (100 mg/kg p.o.) were administered simultaneously at days $-1, 0, 1, 2$ and 5. Ulcers number (A) and score values (B) were determined. Data are expressed as mean $\pm$ S.E. values from nine rats. Statistical analysis for ulcers: one-way analysis of variance followed by Student–Newman–Keuls test; statistical significance ($P < 0.05$), A vs. D; * ($P < 0.05$), C vs. D; ** ($P < 0.01$), D vs. E; *** ($P < 0.001$), A vs. C, D vs. E, **.

ineffective toward the intestinal damage produced by indomethacin.

methacin: the ulcer number and score values are not statistically different in these two groups. In the presence of capsaicin, MED15 loses its protective effect, as demonstrated by the number of ulcers (indomethacin + capsaicin: 69.0 ± 5.4 ; indomethacin + capsaicin + MED15: 61 ± 4.9 n.s.) and by the score values (indomethacin + capsaicin: 7.41 ± 0.36 and indomethacin + capsaicin + MED15: 6.49 ± 0.42 n.s.). In this experiment, MED15, administered in absence of capsaicin, 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).

3.5. 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 antioxidant effects than 5-aminosalicylic acid (MED15 -27.2% , $P < 0.05$; 5-aminosalicylic acid -10.7% n.s.) (Fig. 8).

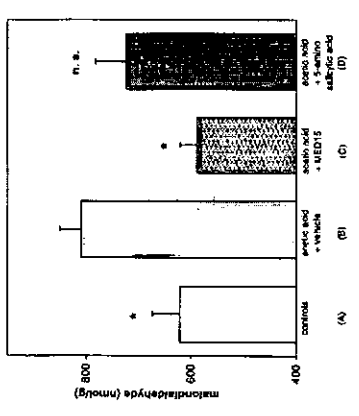


Fig. 8. Effect of MED15 on malondialdehyde levels in acetic acid-induced colitis in the rat. MED15 (100 mg/kg p.o.) and 5-aminosalicylic acid (400 mg/kg p.o.) were administered at 48, 24 and 3 h before the installation of 1% acetic acid (1.5 ml/100 g) into the lumen of the distal colon. Malondialdehyde levels were determined 24 h after induction of colitis. Data are expressed as mean $\pm$ 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 Szecsei, 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 vanilloid moiety of the MED15 molecule, elicited evidence of a protective effect on gastric mucosa in the rat (Whittle et al., 1995; Türoba et al., 2000) and of misoprostol-like activity in human volunteers (Rozzato 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 (Marciolongo et al., 1999).

Due to the strong and persistent presence of the molecule as such in the walls throughout the gastrointestinal 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; Szecsei 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 capsaizine, 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 vanilloid 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 (Mansak and Bauer, 1988). The impact of MED15 on the isolated ileum stimulated with 5-hydroxytryptamine, known to control gastrointestinal motility (Conella, 1981), may be attributable to the efficacy of capsaicin in inhibiting the effects of 5-hydroxytryptamine

(Virta 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 antispasmodic 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 antispasmodic effect of MED15, similar to belladonna alkaloids, mefenazine and pirenzepine, verified using acetylcholine (which increases peristalsis and contraction breadth of the gastroenteric tract) (Fox-Orenstein and Grider, 1996), support possible use of antileptin gureyl 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 (Lewis and Belach, 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 (Kucharik 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 (Cizocova et al., 2001); MED15 shrunk 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 insofar 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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