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The intestinal anti-inflammatory effects of the novel agent UR-1505 in the TNBS model of rat colitis are mediated by T-lymphocyte inhibition

Elvira Bailón [a], *Desirée Camuesco* [a], *Ana Nieto* [b], *Angel Concha* [c], *Alberto Fernández de Arriba* [d], *Juan Román* [d], *Isabel Ramis* [d], *Manuel Merlos* [d], *Antonio Zarzuelo* [a], *Julio Gálvez* [a,1], *Mònica Comalada* [a,1,*]

[a] *Department of Pharmacology, CIBER-EHD, School of Pharmacy, University of Granada, Spain*
[b] *Andalusian Stem Cell Bank, Health and Progress Foundation, Granada, Spain*
[c] *Department of Pathology, Hospital Universitario "Virgen de las Nieves", Granada, Spain*
[d] *Palau Pharma, S.A., Pharmacology & Toxicology, Palau-solità i Plegamans, Barcelona, Spain*

ARTICLE INFO

Article history:
Received 14 May 2007
Accepted 18 July 2007

Keywords:
Salicylates
Cyclosporine A
Th1 inflammation
Crohn's disease
Lymphocyte
Inflammatory bowel disease

ABSTRACT

UR-1505 is a novel pentafluoropropoxy derivative of salicylic acid, selected from a series of salicylate derivatives, according to their activity as inhibitors of T-lymphocyte activation. This study describes the anti-inflammatory activity of UR-1505 on trinitrobenzenesulphonic acid-induced colitis in rat, an experimental model that resembles to Crohn's disease (CD), as well as its *in vitro* effects on T-cells and bone marrow-derived macrophages (BMDM) activation. UR-1505 showed intestinal anti-inflammatory effect, associated with reduced colonic levels of TNFα and LTB_4, inhibition of the expression of IFNγ and iNOS, and lower colonic leukocyte infiltration. The *in vitro* assays revealed that UR-1505 also inhibited T-lymphocyte proliferation and IL-12/IFNγ production, two of the main pro-inflammatory cytokines involved in the pathogenesis of CD. However, UR-1505 did not modify LPS- nor IFNγ-induced activation in BMDM. Thus, UR-1505 specifically affects T-cells without modifying the activation of BMDM. In conclusion, the intestinal anti-inflammatory activity of UR-1505 seems to be mediated by a reduction in the recruitment of immune cells to the inflammatory foci, together with the inhibition of T-cell activation. These results suggest that UR-1505 may be an interesting candidate to be explored for the treatment of CD.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract that comprises two major conditions: Crohn's disease (CD) and ulcerative colitis (UC). Although the pathogenesis of IBD remains elusive, an exacerbated chronic activation of the immune and inflammatory cascade in genetically susceptible individuals may

* *Corresponding author at*: Department of Pharmacology, School of Pharmacy, University of Granada, Campus Universitario 'La Cartuja' s/n, 18071 Granada, Spain. Tel.: +34 958243889; fax: +34 958248964.
E-mail address: comalada@ugr.es (M. Comalada).

[1] Both authors contributed equally to the supervision of this study.

Abbreviations: BMDM, bone marrow-derived macrophage; CsA, cyclosporine A; Con A, concanavalin A; COX, cyclooxygenase; CD, Crohn's disease; GSH, glutathione; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; LTB_4, leukotriene B_4; MPO, myeloperoxidase; SA, salicylic acid; TNBS, trinitrobenzenesulphonic acid; UC, ulcerative colitis.

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doi:10.1016/j.bcp.2007.07.026

play a key role [1]. This response is characterized by an up-regulation of the synthesis and release of a variety of proinflammatory mediators, including eicosanoids, platelet activating factor, reactive oxygen and nitrogen metabolites and cytokines, that hazard mucosal integrity and lead to excessive tissue injury [2,3]. This initial inflammation may lead to the activation of either a Th1 T-cell response, associated with excessive IL-12/IFNγ/TNFα secretion, or a Th2 T-cell response, associated with increased IL-4/IL-5 secretion [4], which will be responsible for the exacerbation or perpetuation of the inflammatory process.

To investigate IBD pathogenic mechanisms, several experimental models of gut inflammation have been described, being the majority of them characterized by the development of an increased Th1 response [5]. One of the most used is the TNBS (trinitrobenzenesulphonic acid) model of rat colitis, which induces transmural cellular infiltration lesions similar to CD [4,5]. In fact, CD has been proposed to be mainly a Th1-mediated inflammation [6,7], and this has been supported by the increased production of IFNγ, IL-12 and TNFα observed in the inflamed intestinal tissue.

Nowadays, a specific causal treatment of CD is not available yet, since the predisposing and trigger factors have not been clearly identified. Therefore, therapeutic and preventive strategies for these disorders must rely on interrupting or inhibiting the immunopathogenic mechanisms that are involved. One of the strategies to effectively down-regulate the exacerbated immune response that characterizes this intestinal condition may be to interfere with the initial steps in this immune response by blocking T-cell activation and proliferation. In fact, several immunomodulator agents currently used for the management of human IBD, such as azathioprine, methotrexate, cyclosporin A or tacrolimus, act by inhibiting T-cell activation [8]. However, the administration of these drugs is limited either by inconstant efficacy, a relatively slow onset of action, inadequate selectivity or by substantial short- and long-term toxicity [8]. For this reason, the development of new drug treatments that combine efficacy and safety is an important goal in IBD therapy. In this sense, other biological therapies such as infliximab, a chimeric monoclonal anti-TNFα antibody, have been successfully developed for IBD treatment in CD patients [9,10]. In addition, anti-IL-12, anti-IFNγ, anti-IL-6 receptor, or toxin-conjugated anti-IL-7 receptor, are also currently being investigated [reviewed in 9].

Salicylates have been used for long time as a therapy for IBD, and they have been shown to be useful in managing mild-to-moderate active UC and mild active CD, as well as in maintaining remission [11]. These compounds are chemically related with the nonsteroidal anti-inflammatory drugs (NSAIDs), whose effects have been entirely attributed to the inhibition of cyclooxygenase (COX) activity and thus, the synthesis of prostaglandins. However, it has been suggested that additional mechanisms underlie some of their anti-inflammatory actions; in fact, aspirin has been reported to block T-cell activation, although these effects were only observed at relatively high concentrations [12,13]. Furthermore, 5-aminosalicylic acid (5-ASA) is widely used in the treatment of the inflammatory bowel disease and is believed to act through the downregulation of the NF-κB pathway [14].

The aim of this study was to test the intestinal anti-inflammatory properties of a new salicylate derivative, UR-1505 (2-hydroxy-4-(2,2,3,3,3-pentafluoropropoxy)-benzoic acid). In this sense, our results show that UR-1505 has intestinal anti-inflammatory activity, mainly due to the inhibition of lymphocyte activity. In addition, the ability of UR-1505 to reduce several inflammatory colonic markers *in vivo*, such as TNFα and LTB_4 production as well as iNOS and IFNγ expression, seems to be a consequence of a reduced recruitment of immune cells to the inflammatory foci and/or to the inhibition of T cell activation thus interrupting the perpetuation of the inflammatory response involved in a colitic process.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma Chemical (Madrid, Spain), unless otherwise stated. Glutathione reductase was provided by Boehringer Mannheim (Barcelona, Spain). UR-1505 (2-hydroxy-4-(2,2,3,3,3-pentafluoropropoxy)-benzoic acid) was provided by J. Uriach y Compañía S.A. (Palau-solità i Plegamans, Barcelona, Spain).

2.2. Animals

Animals were obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) and housed in a temperature (22 °C) and light-controlled (12 h) cycle. This study was carried out in accordance with the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the European Union (86/609/EEC) in compliance with the Helsinki declaration.

2.3. Induction of colitis and assessment of colonic damage

After a 7-day acclimation period, 40 female Wistar rats (180–200 g) were weighed and randomly distributed in the different experimental groups ($n = 10$ per group). Rats were fasted overnight and those from the TNBS control and treated groups were rendered colitic by the method originally described by Morris et al. [15], with some modifications established by our group [16,17]. Briefly, they were anaesthetized with halothane and given 10 mg of TNBS dissolved in 0.25 mL of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically 0.25 mL of phosphate buffered saline instead of TNBS. Two of the colitic groups received by oral gavage 10 or 30 mg/kg/day of UR-1505 dissolved in a vehicle consisting of Tween 80 (1%, v/v) in distilled water, starting the same day of colitis induction. The other two groups, non-colitic and TNBS control, received daily the vehicle used to administer the test compound. All rats were sacrificed with an overdose of halothane 1 week after induction of colitis, and during the experiment, animal body weight, occurrence of diarrhea and food intake were recorded daily.

Once the rats were sacrificed, the colon was removed and placed on an ice-cold plate, cleaned of fat and mesentery and blotted on filter paper; each specimen was weighed and its length measured under a constant load (2 g). Afterwards, the colon was opened longitudinally and scored for macroscopically visible damage on a 0–10 scale according to the criteria described by Bell et al. [18]. Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the segment adjacent to the gross macroscopic damage and were fixed in 4% buffered formaldehyde for the histological analysis. Equivalent colonic segments were also obtained from the non-colitic group. The colon samples were subsequently sectioned in different longitudinal fragments to be used for biochemical determinations and for RNA isolation.

Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al. [19]; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide/min at 25 °C. Total glutathione content was quantified with the recycling assay described by Anderson [20], and the results were expressed as nmol/g wet tissue.

2.4. Immunohistological analysis

The immunolabelling was performed on formalin-fixed paraffin wax-embedded samples using the streptavidin-biotin peroxidase complex method and a high temperature pretreatment as antigen unmasking protocols. After pretreatment, sections were incubated 15 min with 1% hydrogen peroxidase to block endogenous peroxidase activity and were preincubated with protein blocking sera for 30 min to reduce non-specific reactions. The indirect immunofluorescence methods were performed according to established procedures. The primary mouse monoclonal antibodies used were anti-rat mononuclear phagocytes (BD Pharmingen, clone 1C7), anti-rat granulocytes (BD Pharmingen, clone RP-1) and anti-rat CD3 (BD Pharmingen, clone G4.18). Primary antibodies were diluted 1:40 in TBS (Tris buffered saline) and incubated in a humidity chamber overnight at 4 °C. Samples were later incubated with appropriate FITC-conjugated secondary antibodies (Dako Co. Inc.) diluted 1:30 at 37 °C. Sections were then mounted with Aquatex© (Merck and Co. Inc.).

2.5. Analysis of IFNγ expression in colonic samples by RT-PCR

The treatment of colonic samples was measured using minor modifications of a semiquantitative RT-PCR technique previously used in skin biopsies [21]. Total RNA from colonic samples was isolated using TRIzol® Reagent (Gibco-BRL, USA) following the manufacturer's protocol. cDNA was synthesized using First-Strand cDNA Synthesis Kit (Amersham, Biosciences). The primer sequences were: IFNγ (forward 5′-GCTTTGCAGCTCTTCCTCAT-3′, reverse 5′-GCTGATGGCCTGATTGTCTT-3′), β-actin (forward 5′-AATCGTGCGTGACATCAAAG-3′, reverse 5′-ATGCCACAGGATTCCATACC-3′). The polymerase chain reaction was performed as described previously [22] but in this case, the range of cycles were; 38 cycles for IFNγ and 23 cycles for β-actin.

2.6. Cell cultures

Spleens from Balb/C mice (20–24 g) were removed and homogenized in Dulbecco's modified Eagle's medium (DMEM) plus 1% penicillin/streptomycin. After centrifugation (1500 rpm, 5 min) erythrocytes were lysed with the buffer (NH_4Cl 1.7 mol/L, $KHCO_3$ 0.12 mol/L, ethylenediamine-tetraacetic 9 mmol/L) for 30 min at 4 °C. Resting cells were counted by using a hemacytometer and cultured to perform proliferation and stimulation assays in current culture medium containing DMEM plus 10% fetal bovine serum (FBS). Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Bone marrow-derived macrophages (BMDM) were obtained from the same mice and cultured in DMEM which contained 20% FBS and 30% L-cell-conditioned media as a source of macrophage-colony stimulating factor (M-CSF) [23]. After 7 days of culture, a homogeneous population of adherent macrophages was obtained. To render cells quiescent, at 80% confluence macrophages were deprived of L-cell-conditioned media for 16–18 h before the distinct stimuli [23].

2.7. Proliferation assays

Spleen derived lymphocytes were cultured in 24-well plates (1.5×10^6 cells/well) in 0.5 mL of media and stimulated with concanavalin A (Con A) (5 μg/mL). Bone marrow-derived macrophages were cultured in 24-well plates (1×10^5 cells/well) in 0.5 mL of media and stimulated with L-cell. Cell proliferation was measured by [^{3}H] thymidine incorporation as previously described [23] in presence or absence of the indicated concentrations of different compounds.

2.8. Viability assay

The splenocyte viability was quantified using the WST-1 Reagent (Roche, Germany) following the manufacturer's protocol. Viability of macrophages was measured using a crystal violet technique as previously described by Comalada et al. [24] with minor modifications. Macrophages were plated in 24-well culture plates at a confluent condition and were treated with the indicated UR-1505 and salicylic acid (SA) concentrations. The treated cells were maintained at 37 °C in 5% CO_2 for 24 h and then stained and fixed with 0.2% crystal violet in 2% ethanol during 30 min at room temperature. After four washes with PBS, the cells were scrapped with SDS 1% during 30 min and then collected and centrifuged at 3000 rpm during 5 min. Finally, the colour intensity was quantified using an ELISA reader at 540 nm. Each assay condition was performed in triplicate and the results were represented as the mean (% of cell viability) ± S.D.

2.9. Determination of cytokine production

Splenocytes and macrophages were cultured in 24-well plates in the presence or absence of different UR-1505, SA and cyclosporine A (CsA) concentrations as indicated in figures for 1 h before the stimulation with Con A (5 μg/mL) or LPS (10 ng/mL). Supernatants were obtained 24 h (BMDM) or 48 h (T-lymphocytes) after and frozen until ELISA analysis. Cytokine production was measured with murine ELISA kits

(Cytosets™, Biosource International, Nivelles, Belgium) following the manufacturer's protocol.

The TNFα and LTB_4 determinations in colonic samples were analyzed as previously described [25].

2.10. Western blot analysis

The iNOS and COX-2 Western blots from tissue and cells were performed as described elsewhere [26]. Equal amounts of protein from tissue samples (150 μg) and cells (70 μg) were separated on 7.5% SDS-PAGE. For iNOS (Transduction Laboratories, BD Biosciences, Madrid, Spain) and COX-2 (Cayman Chemical Company, MI, USA) we used 1/3000 antibody dilution. A primary antibody against β-actin was used as loading control. Peroxidase-conjugated anti-rabbit IgG were used as secondary antibodies. Then, ECL (Perkin Elmer™, Life Sciences, Boston, USA) detection was performed. The quantification of bands was performed by densitometric analysis using Scion Image program.

2.11. Statistical analysis

In vivo results are expressed as mean ± S.E.M., except for non-parametric data (score) that are expressed as median (range). *In vitro* results are expressed as mean ± S.D. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA) and post hoc least significance tests. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at $P < 0.05$.

3. Results

3.1. The inhibitory effect of UR-1505 on cellular proliferation is specific for T-lymphocytes

The first aim of this study was to evaluate the specificity of UR-1505, a new salicylic acid derivate. Since it has been reported that acetylsalicylic acid blocks T-cell activation [13], we evaluated the effects of UR-1505 and salicylic acid (SA), which was used as a control, on the *in vitro* proliferation of murine splenocytes activated with concanavalin A (Con A). To determine the involvement of UR-1505 and SA in the proliferative response we used ^{3}H-thymidine incorporation as an indicator of cell proliferation [23]. Con A (5 μg/mL) induced T-lymphocyte proliferation (Fig. 1A), and this was inhibited dose-dependently by UR-1505, achieving a complete

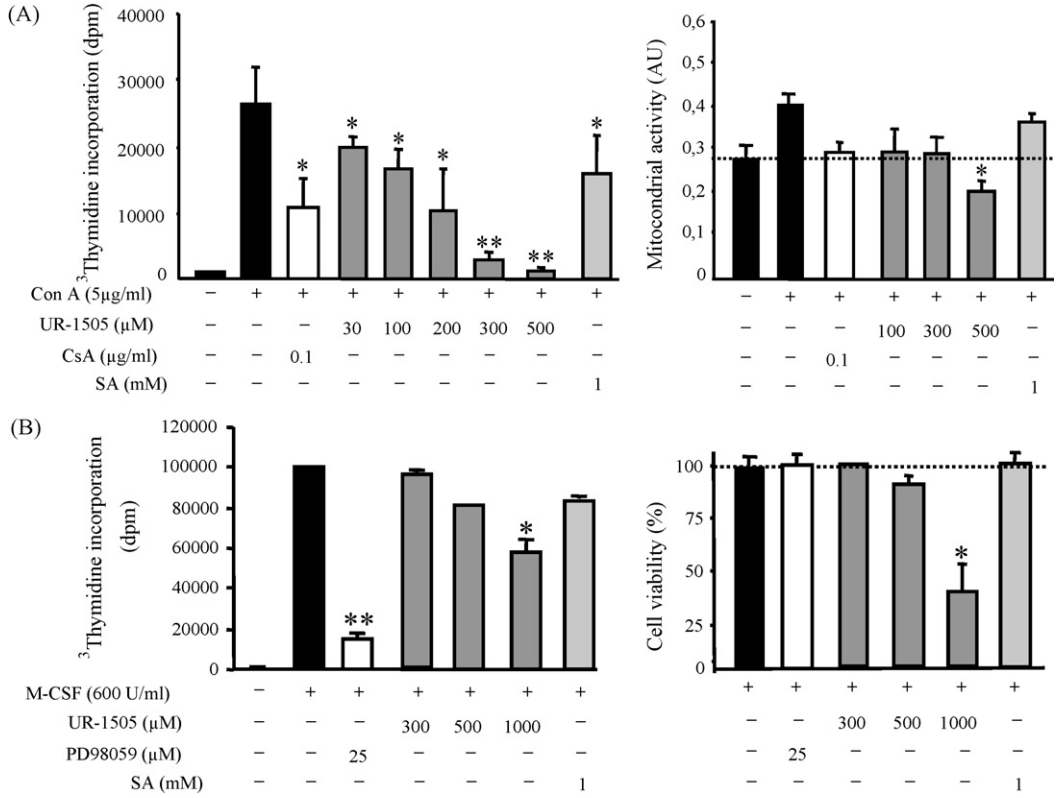

Fig. 1 – Effect of UR-1505 on cellular proliferation and viability on T-lymphocytes and macrophages. (A) UR-1505 inhibits the proliferation of Con A activated T-lymphocytes. Splenocytes were incubated in the presence of the indicated amounts of UR-1505, CsA and SA, and stimulated with Con A (5 μg/mL) during 48 h. Proliferation was determined by ^{3}H-Thymidine incorporation and cellular viability by WST-1 reagent as indicated in Section 2 ($^*P < 0.05$, $^{**}P < 0.01$ vs. Con A). (B) UR-1505 at physiologic concentrations does not inhibit the proliferation and viability in macrophages. BMDM were incubated with M-CSF in the presence or absence of indicated concentrations of UR-1505, SA and PD98059 for 24 h. Proliferation was determined by ^{3}H-Thymidine incorporation and cellular viability by Crystal violet technique ($^*P < 0.05$, $^{**}P < 0.01$ vs. M-CSF). Results are represented as means ± S.D. of triplicates.

inhibition at the concentration of 300 μM (Fig. 1A). The inhibitory effect of SA on T-lymphocyte proliferation was only observed at the highest concentration tested (1 mM), as described by other authors [27] (Fig. 1A). Additionally, we used the cyclosporin A (CsA) (0.1 μg/mL) as a control drug which is known that inhibits T-lymphocyte proliferation (Fig. 1A). We also examined the influence of UR-1505, SA and CsA on lymphocyte viability using the WST-1 mitochondrial assay. After 48 h of stimulation with Con A, a higher mitochondrial activity was observed in the culture, most probably due to an increase in the cell number as a consequence of the proliferation process rather than to a direct effect on cell viability (Fig. 1A). UR-1505 (100 and 300 μM) reduced mitochondrial activity to basal levels (Fig. 1A), suggesting that these concentrations did not affect T-lymphocyte viability and the observed reduction in this activity may be attributed to its antiproliferative effects. Similar results were obtained after CsA addition to the cell culture (Fig. 1A). On the contrary, SA did not affect the mitochondrial activity of Con A-activated lymphocytes, as it would be expected from its lack of any significant effect on T-cell proliferation.

In contrast to the effects on T-lymphocytes, UR-1505 had only a weak effect on M-CSF-induced macrophage proliferation, at concentrations ranging from 300 μM to 1 mM, since only a 30% inhibition was detected at the highest concentration (Fig. 1B). Similarly, SA (1 mM) did not affect this cell proliferation, but PD98059 (25 μM), a well known inhibitor of macrophage proliferation [23], showed a clear inhibitory activity (Fig. 1B). In addition, macrophage viability was assayed by crystal violet staining (Fig. 1B) and by Trypan Blue (data not shown). The viability was only affected by the highest concentration of UR-1505 assayed (1 mM), and not by lower concentrations, which showed no significant effect on macrophage proliferation although they effectively inhibited T-lymphocyte proliferation. Similarly, SA did not modify macrophage viability even at high concentrations (1 mM) (Fig. 1B).

3.2. *Inhibitory effects of UR-1505 on cytokine secretion in activated T-lymphocytes and macrophages*

Stimulation of splenocytes with Con A induces the secretion of different cytokines, including IFNγ (Fig. 2A), IL-12 (Fig. 2B) and TNFα (Fig. 2C). These cytokines are typically secreted after activation of Th1 lymphocytes. The TNBS experimental model of rat colitis is also characterized by an overproduction of these Th1 cytokines [6,7]. The pre-treatment with UR-1505 inhibited Con A-induced IFNγ and IL-12 production, in a concentration-dependent manner, showing a 50 and 100% inhibition (Fig. 2A and B), respectively, at 300 μM. However, TNFα secretion was not significantly inhibited by UR-1505. CsA drastically downregulated both IFNγ and TNFα secretions, whereas it slightly increased IL-12 secretion (Fig. 2A, B and C), as previously reported [28]. SA did not significantly modify IFNγ or TNFα secretions in activated T-lymphocytes, although it was able to increase IL-12 production (Fig. 2B). These results were confirmed by RT-PCR for the different cytokines analyzed (data not shown).

UR-1505 and SA were also tested in LPS- or IFNγ-activated bone marrow-derived macrophages (BMDM). LPS induced high

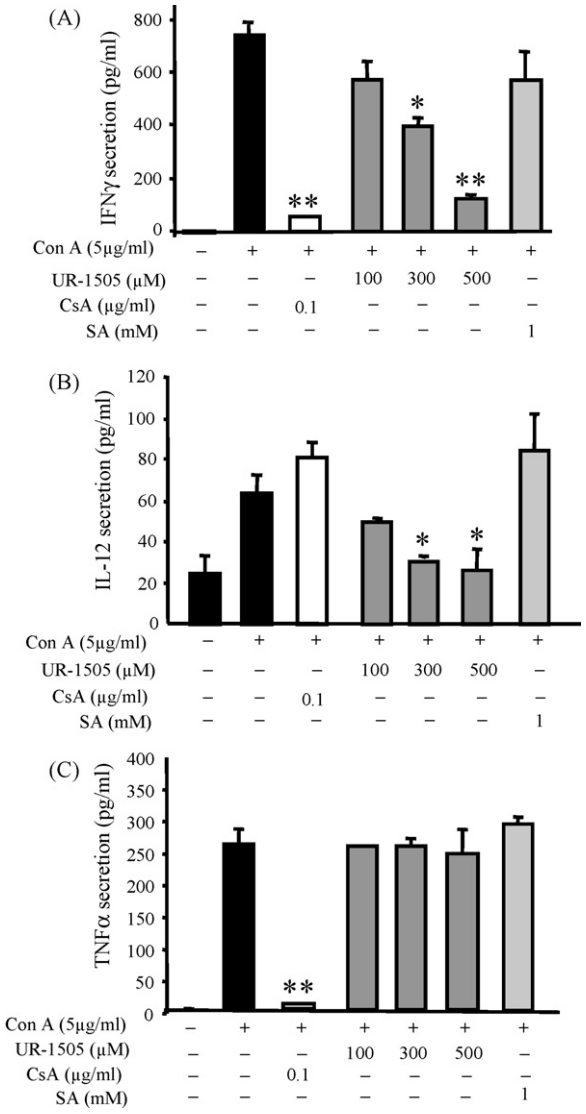

Fig. 2 – Effect of UR-1505 on Th1 cytokine secretion in activated T-lymphocytes. UR-1505 inhibits IFNγ (A), IL-12 (B) but does not affect to TNFα (C) secretion in Con A-activated T-lymphocytes. Splenocytes were incubated with indicated concentrations of UR-1505, CsA and SA and activated with Con A during 48 h. The concentrations of cytokines in the culture supernatants were analyzed by ELISA. The results of a representative experiment of four independent experiments are shown as means ± S.D. of triplicates ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. Con A).

levels of TNFα and IL-12 after 24 h. In these cells, UR-1505 (500 μM) did not downregulate TNFα or IL-12 secretion. SA (1 mM) inhibited TNFα secretion in this cell type (Fig. 3A), whereas it increased IL-12 production (Fig. 3B). Moreover, UR-1505 (100 μM–1 mM) was devoid of any significant effect on iNOS or COX-2 expression after LPS or IFNγ stimulation (Fig. 3C and D). However, SA (1 mM) inhibited the COX-2 expression induced by LPS or IFNγ activation but only induce a minor modification on iNOS expression (Fig. 3C and D).

All these results reveal that UR-1505 specifically affects T-lymphocytes without showing significant effects on macro-

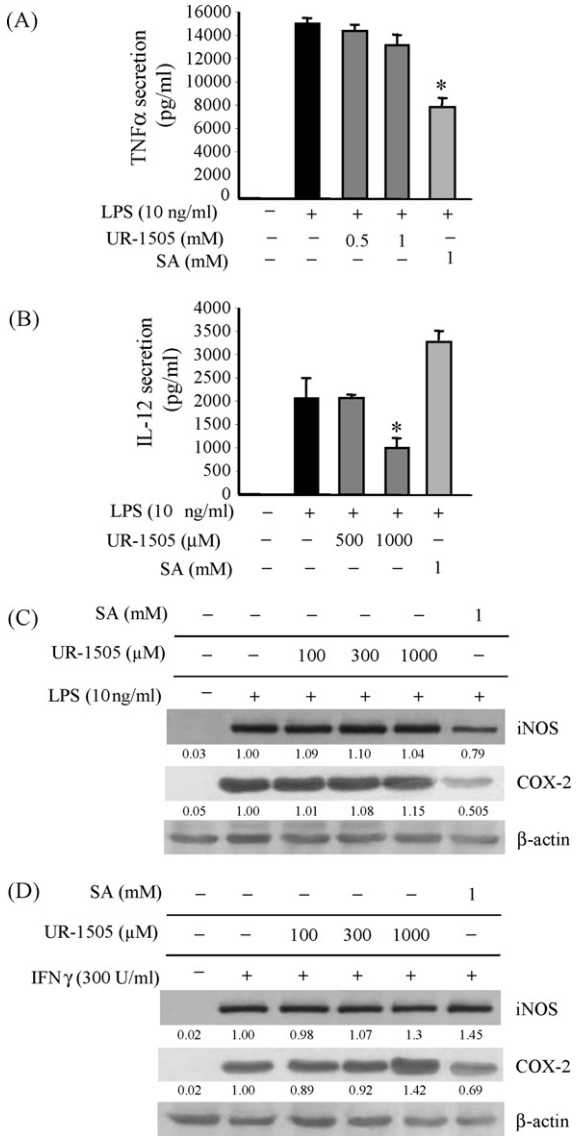

Fig. 3 – Effect of UR-1505 on pro-inflammatory markers in activated macrophages. UR-1505 at high doses does not affect to TNFα secretion (A), but inhibits IL-12 secretion (B) in LPS-activated macrophages. BMDM were incubated with indicated concentrations of UR-1505 and SA and then were activated with LPS during 24 h. The concentrations of cytokines in the culture supernatants were analyzed by ELISA. Each experiment was performed three times and represented as the mean ± S.D. UR-1505 does not inhibits the iNOS and COX-2 expression induced by LPS (C) or IFNγ (D) in macrophages. BMDM were pretreated with UR-1505 or SA for 1 h and then incubated with LPS or IFNγ for 6 and 24 h, respectively. The iNOS and COX-2 expression were analyzed by Western blotting. Densitometric analysis of the bands was performed and the values obtained are represented in the figure as the normalized band intensity (iNOS or COX-2/actin) and referred as the maximum intensity band value in LPS or IFNγ stimulated value (1.00). All of these experiments were performed in triplicate with similar results ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS).

phage activity. Low concentrations of this drug (100–300 μM) inhibited activated T-cell proliferation and the production of different cytokines. On the contrary, even high concentrations did not significantly modify TNFα secretion, COX-2 or iNOS expression in activated macrophages.

3.3. *Inhibitory effect of UR-1505 on inflammation in the TNBS model of rat colitis*

The second aim of this work was to address whether UR-1505 inhibits the inflammation induced in the TNBS model of rat colitis. The assayed doses of UR-1505 were 10 and 30 mg/kg. One week after colitis induction, thickening of the bowel wall was evident in TNBS control rats with a significant increase in the colonic weight/length ratio in comparison with non-colitic rats ($P < 0.01$; Table 1). The administration of UR-1505 (10 or 30 mg/kg) for 7 days exerted an intestinal anti-inflammatory effect that included a decrease in the weight/length ratio in UR-1505 treated colitic groups compared to TNBS control ($P < 0.05$; Table 1), as well as a significant reduction in the extent of colonic damage, resulting in a lower macroscopic damage score (Table 1). The intestinal anti-inflammatory activity of UR-1505 was also evidenced biochemically since it significantly counteracted glutathione depletion that occurred as a consequence of the inflammation in untreated colitic rats (Table 1). Of note, preliminary experiments performed in healthy rats treated with UR-1505 for 7 days, at doses ranging from 5 to 30 mg/kg, revealed the absence of any deleterious effect when compared with untreated rats (data not shown).

Microscopically, TNBS caused the typical inflammatory features in the colonic architecture: ulceration, crypt dilation, goblet cell depletion as well as mixed cell infiltration, mainly composed by mononuclear cells (macrophages, lymphocytes and plasmatic cells), although granulocytes were also present (Fig. 4B). Both doses of UR-1505 improved the colonic architecture since most of the treated rats showed an almost complete restoration of the damaged colon displaying a moderate inflammatory infiltrate with a focal distribution (Fig. 4C and D). Only 20% of the treated rats presented of gross ulceration with a prominent inflammatory cell infiltrate. There was also a restoration in the crypt architecture with goblet cell and mucin replenishment (Fig. 4).

3.4. *UR-1505 treatment down-regulates several pro-inflammatory markers in the TNBS model of rat colitis*

We further evaluated the effects of oral administration of UR-1505 (10 and 30 mg/kg/day) on several colonic inflammatory markers in the TNBS model of rat colitis 7 days after, i.e. colonic TNFα and LTB_4 production, myeloperoxidase (MPO) activity, as well as iNOS, COX-2 and IFNγ expression, being the latter one of the main pro-inflammatory cytokines in CD [6,7]. As expected, all these biochemical parameters were significantly modified as a consequence of the inflammatory process, showing increased colonic MPO activity, LTB_4 and TNFα levels (Table 2), as well as enhanced iNOS (Table 2 and Fig. 5A), COX-2 (Fig. 5A) and IFNγ (Fig. 5B) expression. UR-1505 treatment, at doses of 10 and 30 mg/kg, significantly inhibited MPO activity, confirming the decrease of neutrophil infiltration evidenced histologically (Fig. 1). Moreover, UR-1505

Table 1 – Effects of UR-1505 (10 and 30 mg/kg) treatment in colon weight/length, extent of the inflammatory lesion along the colon, macroscopic damage score and glutathione (GSH) content in TNBS experimental colitis in rats

Group ($n = 10$)	Weight/length (mg/cm)	Extent of damage (cm)	Damage score (0–10)	GSH (nmol/g)
Non-colitic	61.2 ± 1.7	0	0	1948 ± 44
TNBS control	$184.2 \pm 13.3^{\#\#}$	$4.7 \pm 0.3^{\#\#}$	7.5 (7–9)$^{\#\#}$	$1652 \pm 55^{\#\#}$
UR-1505 (10 mg/kg)	$155.8 \pm 14.3^{\#\#,*}$	$3.3 \pm 0.5^{\#\#,*}$	6 (4–9)$^{\#\#,*}$	$1835 \pm 59^{*}$
UR-1505 (30 mg/kg)	$142.2 \pm 13.6^{\#\#,*}$	$2.7 \pm 0.4^{\#\#,**}$	6 (4–7)$^{\#\#,**}$	$1854 \pm 60^{*}$

The colon weight data and extent of damage are expressed as mean ± S.E.M.
* $P < 0.05$ vs. TNBS control group.
** $P < 0.01$ vs. TNBS control group.
$^{\#\#}$ $P < 0.01$ vs. non-colitic group. Damage score for each rat was assigned according to the criteria described by Bell et al. (1995) and data are expressed as median (range).

administration to colitic rats resulted in a significant reduction of the colonic production of LTB_4 and TNFα (Table 2). The beneficial effects exerted by both doses of UR-1505 on colonic inflammation were also associated with a lower colonic expression of iNOS (Table 2 and Fig. 5A) and IFNγ (Fig. 5B) when compared with TNBS control animals. However, UR-1505 treatment was not able to inhibit the COX-2 expression induced by TNBS administration (Fig. 5A).

3.5. *UR-1505 treatment affects cell infiltration into the intestinal epithelium in the TNBS model of rat colitis*

Immunofluorescence staining of colonic samples from TNBS control group confirmed the presence of both macrophages and T-lymphocytes in the inflamed areas of the intestine, mainly located in the lamina propria and the submucosa adjoining regions of ulceration (Fig. 6). In addition, neutrophils were also found in crypt abscesses and in the surface of ulcerated epithelium (data not shown). The intestinal anti-inflammatory effect exerted by UR-1505 is associated with a decreased number of infiltrated macrophages, T-lymphocytes (Fig. 6) and neutrophils (data not shown).

4. Discussion

CD is characterized by a chronic-intermittent transmural, segmental, and typically granulomatous inflammation of different segments of the intestine. Although many important findings have been reported recently, it is not fully understood why the mucosal immune response is over-reactive in patients with this intestinal condition, or how current medical treatment affects the mucosal immune system. However, it has become more and more evident that alterations in the

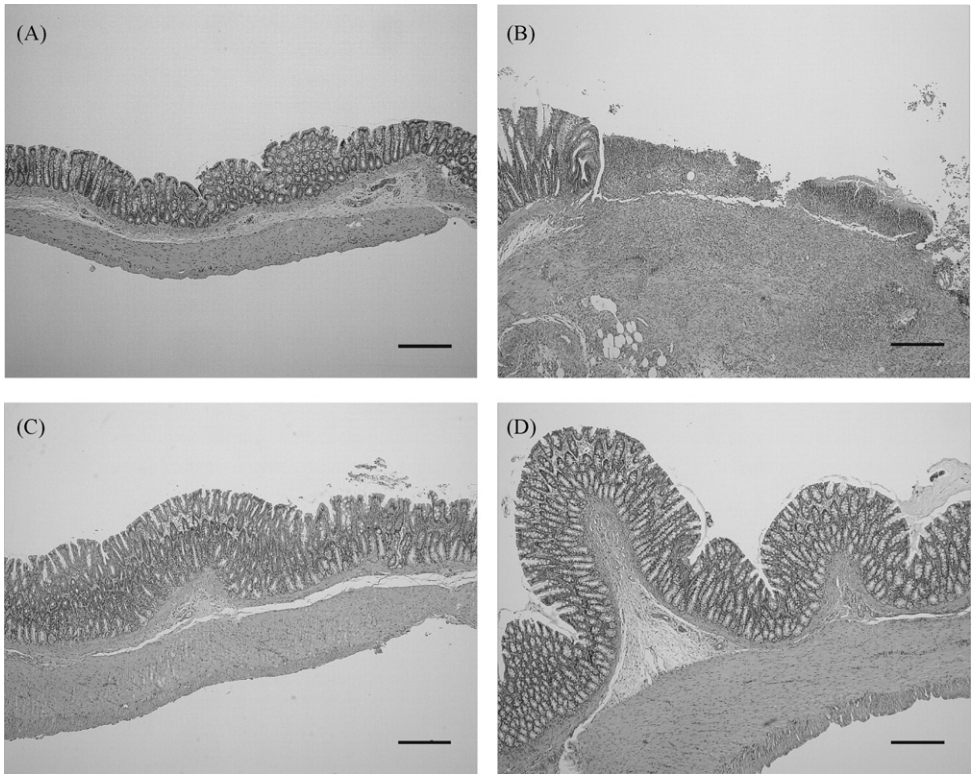

Fig. 4 – Histological sections of colonic mucosa from TNBS-induced colitic rats treated with UR-1505 stained with hematoxylin and eosin showing the anti-inflammatory effect of UR-1505 treatment. (A) Non-colitic group; (B) TNBS-control group; (C and D) UR-1505 treated groups at doses of 10 and 30 mg/kg, respectively. Calibration bar = 200 μm.

Table 2 – Effects of UR-1505 (10 and 30 mg/kg) treatment on colonic MPO activity, LTB_4 and TNFα and iNOS expression in induced-TNBS experimental colitis in rats

Group ($n = 10$)	MPO activity (units/g)	LTB_4 (ng/g)	TNFα (pg/g)	iNOS (arbitrary units)
Non-colitic	27.7 ± 4.5	2.2 ± 0.3	226.5 ± 12.0	0.162 ± 0.018
TNBS control	$181.4 \pm 13.8^{\#\#}$	$7.8 \pm 0.4^{\#\#}$	$309.5 \pm 20.0^{\#\#}$	$1.078 \pm 0.142^{\#\#}$
UR-1505 (10 mg/kg)	$148.0 \pm 9.5^{\#\#,*}$	$5.6 \pm 0.5^{\#\#,**}$	$232.7 \pm 27.9^{*}$	$0.451 \pm 0.038^{\#\#,**}$
UR-1505 (30 mg/kg)	$130.1 \pm 5.6^{\#\#,**}$	$4.9 \pm 0.7^{\#\#,**}$	$204.3 \pm 20.1^{**}$	$0.460 \pm 0.030^{\#\#,**}$

Data are expressed as mean ± S.E.M.
* $P < 0.05$ vs. TNBS control group.
** $P < 0.01$ vs. TNBS control group.
$^{\#\#}$ $P < 0.01$ vs. non-colitic group.

innate immunity are crucial in the pathogenesis of CD and involve a deregulated Th1 lymphocyte response [6,7]. Considering this, different immunosuppressive therapies have been developed lately [9,10,29].

In the present study, we have used the TNBS model of rat colitis that resembles CD pathology [4,5]. Indirect evidences suggest that $CD4^+$ T cells play a major role in the initiation and perpetuation of TNBS colitis, mostly driven by a Th1 cytokine response, as supported by the attenuating effects obtained after T cell-directed immunotherapies: anti-IL-12 monoclonal antibody (mAb), anti-CD40L mAb, antiinterferon-γ mAb, or OX40-Ig [30,31].

UR-1505 is a novel salicylate derivative with an adequate oral bioavailability in rats, since 96.9% of the administered dose (ranging from 10 to 50 mg/kg), is absorbed in the small intestine, thus providing high plasma levels (unpublished data). In consequence, the oral administration of a unique dose of 30 mg/kg would probably result in a blood concentration higher than 200 μM, and in consequence, able to systemically inhibit lymphocyte activity as demonstrated in the *in vitro* experiments.

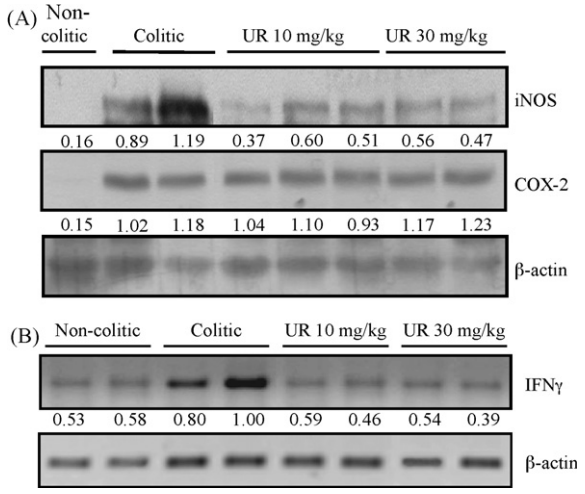

Fig. 5 – UR-1505 treatment inhibits IFNγ and iNOS expression but it does not modify COX-2 expression in TNBS-induced colitic rats. The iNOS and COX-2 expression is evaluated by Western blot (A) and IFNγ expression was assessed by semiquantitative RT-PCR (B). Densitometric analysis of the bands was performed and the values obtained are represented in the figure as the normalized band intensity (iNOS/actin, COX-2/actin and IFNγ/actin) and referred as the maximum intensity band value in control colitic rats (1.00). The gel shown is representative of all colonic samples per group ($n = 10$).

The main aim of this work was to evaluate its anti-inflammatory and immunomodulatory properties in the main types of cells involved in the TNBS rat colitis, namely macrophages and T-lymphocytes, comparing its pharmacological profile with CsA and SA. The *in vitro* results obtained in the present study suggest that UR-1505 inhibits T-cell proliferation and Th1 cytokine production (IL-12 and IFNγ) when T-lymphocytes are activated with Con A. However, this drug neither affects TNFα and IL-12 secretion nor iNOS and COX-2 expression in activated macrophages. For this reason, UR-1505 seems to be more specific for T-lymphocytes than for macrophages, most probably by interfering a common pathway implicated in IL-12 and IFNγ production in activated T-cells. The possible mechanisms involved in this effect are currently being investigated in our group.

UR-1505 inhibits activated T-lymphocyte proliferation in a similar way as CsA. However, there are some differences when their effects on cytokine release are analyzed suggesting different mechanism of action of both drugs; while UR-1505 reduces both IFNγ and IL-12 secretions, without showing any significant effect on TNFα, CsA downregulates IFNγ and TNFα, as well as it slightly increases IL-12 production in Con A activated T-lymphocytes. Although UR-1505 is a derivative of SA, its immunomodulatory properties are also clearly different. In fact, SA has antiproliferative properties in Con A activated T-lymphocytes (although at higher concentrations than UR-1505), but it does not inhibit IFNγ and TNFα secretions, and increases IL-12 production. In addition, SA inhibits COX-2 expression in macrophages stimulated with LPS and IFNγ while UR-1505 does not. All these results suggest that UR-1505 shows a different anti-inflammatory profile than SA, supporting a potential role of this new drug for the oral treatment of different inflammatory conditions associated to an altered immune response such as CD.

The efficacy of UR-1505 in an experimental model of intestinal inflammation has been clearly shown, since this compound was able to facilitate the recovery from TNBS-induced colonic damage, as evidenced by a significant amelioration in the different inflammatory parameters assayed. However, some contradictory results arise between the *in vivo* and the *in vitro* studies regarding the effects of this new drug on the altered immune response. Thus, UR-1505 treatment to colitic rats inhibits the colonic production of TNFα and iNOS expression; however, this drug is not able to

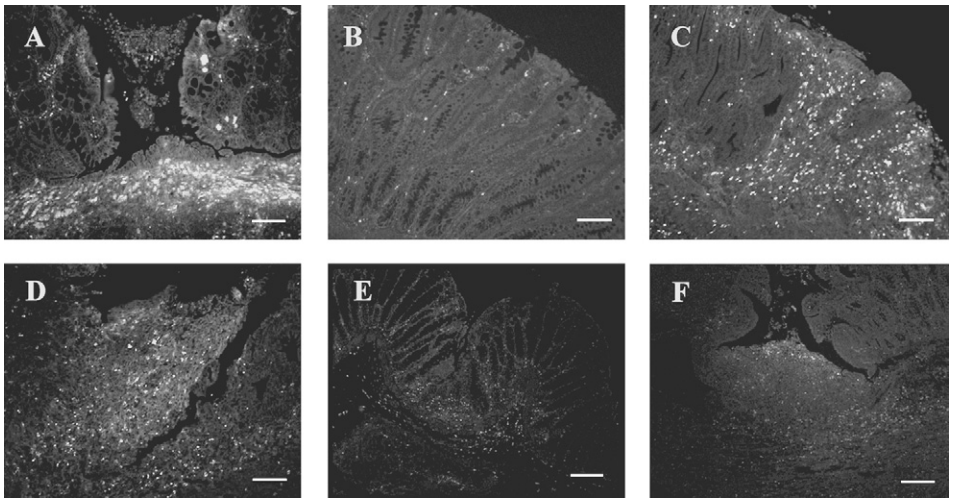

Fig. 6 – UR-1505 treatment inhibits the macrophages and T-lymphocytes recruitment in the TNBS model of rat colitis. Immunofluorescence of macrophages (A–C) and T-lymphocytes (D–F) were performed in histological sections of colonic mucosa from colitic rats. The sections of TNBS-control group (A and D) and UR-1505 treated groups at doses of 10 mg/kg (B and E) and 30 mg/kg (C and F), were incubated with anti-rat mononuclear phagocytes or anti-rat CD3 antibodies. Calibration bar = 50 μm.

downregulate these parameters in activated macrophages, the main producers of these mediators, which are clearly increased in the inflamed intestine [26]. The lower granulocyte infiltration in the damaged areas of the intestine after UR-1505 treatment, as observed biochemically (reduced colonic MPO activity) and by immunohistochemistry, would probably justify the ability of this drug to modify the global inflammatory response, since it is well described that, in the first steps of the gut inflammation, margination and extravasation of circulating granulocytes probably result in the perpetuation of the inflammatory process [32,33]. Supporting this, glutathione depletion also takes place, most probably due to a greater oxidative stress derived from granulocyte activity in the inflamed areas of the intestine [34]. Moreover, the decrease in neutrophil infiltration may also account for the partial reduction in the colonic production of LTB_4 observed after UR-1505 treatment, as neutrophils are considered the main source of this chemotactic eicosanoid in IBD [18].

Although UR-1505 does not probably affect macrophage cell activity *per se*, its oral administration to colitic rats reduces the infiltration of these cells into the inflamed intestine and it may also contribute to its beneficial effects. In fact, macrophages are considered an important source of several pro-inflammatory mediators, such as NO, IL-1β and TNFα [26]. Among these, TNFα secretion and NO overproduction have been proposed to play a deleterious role in colonic inflammation [35–37]. Therefore, we can consider that the *in vivo* inhibitory effects of UR-1505 on colonic TNFα production and iNOS expression might be secondary to a reduced macrophage infiltration, since, as observed *in vitro*, this compound was not able to reduce TNFα production or iNOS expression in activated macrophages. Moreover, although UR-1505 treatment inhibits MPO activity significantly, it does not restore this enzymatic activity to the basal levels. This suggests that UR-1505 does not directly inhibit neutrophil activity (nor macrophage activity) but it is able to reduce the leukocyte infiltration, most probably by inhibiting the production and/or release of chemotactic molecules by other cell types, including lymphocytes. In addition, the lower leukocyte infiltration could also account for the restoration in colonic glutathione, since a lower oxidative insult would occur.

These results led us to suggest that UR-1505 could probably exert its action through the reduction of the inflammatory cell recruitment to the inflammatory foci and the disruption of the positive feedback process involved in the perpetuation of the inflammatory response in the intestine by inhibiting T-lymphocyte activity, proliferation and production and release of cytokines, like IFNγ, the main macrophage activator [38], and IL-12, that promotes the differentiation of CD4+ T-cells into Th1 effector cells [39]. Thus, UR-1505 would downregulate the adaptive immunity and the perpetuation of the inflammatory response.

It is important to note that UR-1505 treatment did not ameliorate COX-2 expression induced by TNBS colitis. This is in agreement with the *in vitro* experiments performed in macrophages when stimulated with LPS or IFNγ, and supports the T-lymphocyte specificity proposed for UR-1505. Although the *in vivo* assays revealed that there was a lower macrophage infiltrate in the colitic group treated with UR-1505, as evidenced by IHC (Fig. 6) or by a reduction in colonic iNOS expression (Fig. 5A), the lack of any significant effect on COX-2 expression may be derived from its different distribution in colonic tissue since it has been proposed that it is mainly induced in intestinal epithelial cells [40], in agreement with the specificity showed by this compound toward T-lymphocytes.

One limitation in this study may arise from the experimental design, since TNBS and UR-1505 were administered concurrently. This may result in an attenuation of the colitis induction by UR-1505 rather than a facilitated recovery of the damaged colonic tissue. In this sense, additional assays performed with this drug revealed that UR-1505 treatment does not prevent the acute colonic damage 24 h after TNBS

administration (data not shown). This suggests that the compound is devoid of any preventative effect in this experimental model of rat colitis, but it facilitates the restoration of the inflamed tissue, an effect already achieved 48 h after TNBS instillation (unpublished results).

In conclusion, UR-1505 administration facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect associated with a reduced recruitment of inflammatory cells in the inflamed tissue that results in the reduction of some proinflammatory mediators, including cytokines, like TNFα and IFNγ, eicosanoids, like LTB_4, and NO. Moreover, its ability to inhibit T-lymphocyte activation also facilitates the recovery of the damaged tissue by inhibiting the reactivation of the exacerbated immune response that takes place in these intestinal conditions, thus suggesting that UR-1505 treatment could be a good candidate for the CD therapy and other Th1-mediated inflammatory diseases. However, clinical studies will be required in order to confirm the safety and efficacy of this new drug in the management of CD.

Acknowledgements

This study was supported by grants from Spanish Ministry of Science and Technology (SAF2005-03199), with funds from the European Union, from Junta de Andalucia (CTS 164) and from J. Uriach y Compañía S.A. (Palau-solità I Plegamans, Barcelona, Spain). M.C. is a recipient of Juan de la Cierva Program from Spanish Ministry of Science and Technology. E.B. is recipient of a fellowship from the Ministry of Science and Technology, Spain. The authors want to thank M.E. Rodriguez-Cabezas for technical assistance.

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