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In vitro inhibition of enterobacteria-reactive CD4+CD25− T cells and suppression of immunoinflammatory colitis in mice by the novel immunomodulatory agent VGX-1027

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Abstract

VGX-1027 is an isozaxoline compound that has recently been found to primarily target the function of murine macrophages but not of T cells, inhibiting secretion of tumor necrosis factor (TNF)-α in response to different Toll-like receptor agonists *in vitro* and *in vivo*. The well-defined role of innate immunity in inflammatory bowel diseases prompted us to consider the use of VGX-1027 in these diseases leading us to *in vitro* and *in vivo* test the drug in related experimental conditions. These consist, respectively, of the proliferation assay of CD4+CD25− T cells to enterobacteria, and the acute inflammatory colitis induced in mice by intracolonic challenge with dinitrobenzene sulfonic acid. The data from the two sets of experiments revealed that VGX-1027 inhibited both proliferation of enterobacterial antigen-reactive CD4+CD25− T cells *in vitro* and the development of clinical and histological signs of colitis *in vivo*. The beneficial effect in this model was associated with reduced colonic production of proinflammatory cytokines such as interleukin (IL)-1β, TNF-α, IL-12p70 and interferon (IFN)-γ and lower content of nuclear factor (NF)-κB (p65). These findings seem to warrant investigations of VGX-1027 for use in human.

Keywords: Cytokines; Inflammatory bowel diseases; Immunotherapy; Nuclear factor-κB VGX-1027

1. Introduction

Crohn's disease and ulcerative colitis are inflammatory bowel diseases that affect different sites of the gastrointestinal tract with more frequent involvement of the ileocaecal region in Crohn's disease and the mucosal layer of the colon in ulcerative colitis. Both diseases are thought to be aetiologically linked to a failure of the mucosal immune system to attenuate immune responses to luminal self or non-self antigens possibly secondary to malfunctioning of CD4+CD25+ regulatory T cells (reviewed by Xavier and Podolsky, 2007; Goyette et al., 2007). This leads to dysregulated activation of nuclear factor(NF)-κB family members that causes an abnormal production of cytokines and cellular infiltration leading to damage of the intestinal mucosa. Although histopathological and immunopathogenic differences occur between Crohn's and ulcerative colitis, the diseases exhibit a similar favourable response to immunosuppressive agents such as corticosteroids, sulfasalazine and azathioprine (Brown and Mayer, 2007). Furthermore, anti-tumor necrosis factor (TNF)-α monoclonal antibodies also favourably influence the course of

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Crohn's disease and ulcerative colitis suggesting that both disease manifestations require endogenous TNF-α as a key pathogenic component (Brown and Mayer, 2007). Unfortunately, long term side-effects related to neutralization of endogenous TNF-α, drug immunogenicity, and the high costs of prolonged therapies limit the use of anti-TNF-α biopharmaceuticals. In addition, these TNF-α inhibitors don't directly interfere with other inflammatory mediators of the innate and adaptive immune systems, for example interleukin (IL)-1β, interferon (IFN)-γ, IL-6, IL-12, IL-18, IL-21 and IL-23 that have also been suggested to play a role in the pathogenesis of inflammatory bowel diseases (Fantini et al., 2007; Brown and Mayer, 2007; Xavier and Podolsky, 2007). Therefore, small molecules that may down-regulate the production of TNF-α and other inflammatory cytokines warrant attention for their possible use in inflammatory bowel diseases.

An additional target for novel therapies of inflammatory bowel diseases is the transcriptional factor NF-κB, the activation of which regulates the production of several proinflammatory cytokines. The relevance of NF-κB in the pathogenesis of inflammatory bowel diseases and the suitability of its inhibitors as potential therapeutic options for the treatment of the disease are supported by the high levels of NF-κB in intestinal mucosa of patients with inflammatory bowel diseases (Schreiber et al., 1998; Rogler et al., 1998) as well as by the anti-inflammatory efficacy of antisense constructs to silence NF-κB (p65) both in animal models of the disease and in the resolution of inflammatory events in human intestinal cells from patients with inflammatory bowel diseases (Neurath et al., 1996).

VGX-1027 ((*S*,*R*)-3-phenyl-4,5-dihydro-5-isoxasole acetic acid) (VGXP, Blue Bell, PA, USA) (see Fig. 1) is an isozaxoline compound that we have previously shown to exerts its immunomodulatory activities *in vitro* by targeting the function of macrophages. VGX-1027 inhibits lipopolysaccharide (LPS)-induced NF-κB, p38 mitogen activated protein (MAP) kinase signaling pathways and TNF-α secretion from purified peritoneal macrophages *in vitro* (Stojanovic et al., 2007). We also demonstrated that VGX-1027 inhibited LPS-induced secretion of IL-1β, TNF-α and IL-10 from peritoneal cells upon *in vitro* and *ex vivo* conditions (Stojanovic et al., 2007). The action of VGX-1027 on macrophages seems to occur through inhibition of TLR4 and TLR2/6-mediated signalling pathways as the test compound inhibited the release of TNF-α from macrophages stimulated with specific agonists of TLR4 and TLR2/6 such as LPS and zymosan, respectively (Stojanovic et al., 2007). In contrast, VGX-1027 appeared to spare T cell function as it did not modify the proliferation and/or secretion of IL-2, IFN-γ, and IL-4 induced in purified murine CD4+T cells from stimulation with either CD3+CD28 or Concanavalin A (ConA) (Stojanovic et al., 2007). These effects on macrophages may account for the capacity of VGX-1027 to markedly ameliorate the course of both acute and chronic immunoinflammatory diseases in rodent models (Stosic-Grujicic et al., 2007; Stojanovic et al., 2007). VGX-1027 is effective both when given i.p. and p.o. and acute and sub-acute toxicological studies show that it is not toxic at the doses that exert biological effects in these preclinical models.

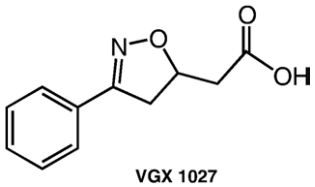

Fig. 1. (*S*,*R*)-3-phenyl-4,5-dihydro-5-isoxasole acetic acid — named VGX1027.

The well known role of innate immunity on the development of inflammatory bowel diseases prompted us to investigate VGX-1027 in *in vitro* and *in vivo* studies in related experimental conditions. These consisted of a proliferation assay of CD4+CD25− T cells challenged by enterobacteria, and acute inflammatory colitis induced in mice by intracolonic challenge with dinitrobenzene sulfonic acid (DNBS) (Hoffman et al., 2002-2003). The data revealed that VGX-1027 inhibited proliferation of enterobacteria-reactive T cells *in vitro* and markedly reduced clinical and histological signs of colitis in DNBS-challenged mice. The beneficial effect in the colitis model was associated with reduced colonic production of the proinflammatory cytokines, IL-1β, TNF-α, IL-12p70 and IFN-γ, and lower contents of nuclear NF-κB (p65).

2. Materials and methods

2.1. Animals

The study was carried out in 6–8 weeks old (25–30 g) CD1 male mice (Harlan Nossan, San Pietro Natisone, Udine, Italy). For the *in vitro* part of the study 6 to 8 week old female BALB/c mice (Harlan Nossan) were used. The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. Preparation of faecal extract

Extracts were prepared by removing the colon and the caecum from the BALB/c mice and placing the content in phosphate-buffered saline (PBS) with 10 mg/ml DNase and 1% vol. glass beads as described by Brines et al. (2001). This mixture was solicited three times 30 s on ice, and then centrifuged at 10,000 ×g for 10 min to remove debris. The supernatant was collected, sterile-filtered and stored −80 °C. The protein concentration in the supernatant was typically 1–1.5 mg/ml as determined by the Bio-Red colorimetric assay according to the method of Bradford (Bio-Red).

2.3. Preparation and pulsing of antigen-presenting cells

Splenocytes (8×10^6 cells/well in 2 ml complete medium in 24 well plates) from BALB/c mice were pulsed overnight with 200 μg/ml enterobacterial extract in the presence or absence of different concentrations of VGX-1027 as shown in Fig. 2, and

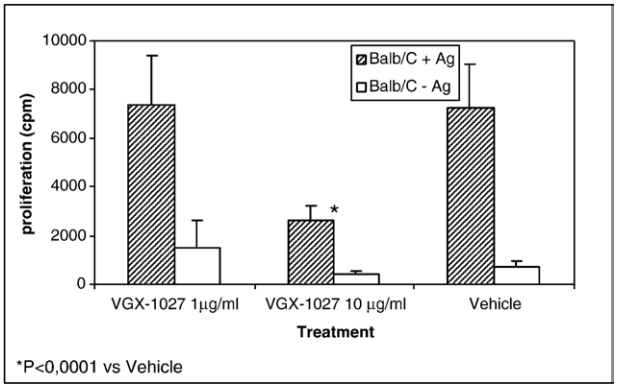

Fig. 2. Effect of VGX-1027 on proliferation of enterobacteria-reactive CD4+ CD25− T cells. Results are from the means of two experiments with 8 mice per group.

irradiated (3000 rad) after incubation for 18 h as previously described (Brines et al., 2001).

2.4. Isolation of CD4+CD25− T cells from BALB/c mice

CD4+ T cells were isolated from eight normal BALB/c mouse lymph nodes and spleens with anti-CD4 Dynabeads and detach-a-beads (Dynal, Oslo, Norway) according to the manufacturer's instruction. These cells were then depleted of CD25+ cells with phycoerythrin-conjugated anti-CD25 and anti-phycoerythrin micro beads kit from MACS Milteny (Bergish, Gladbach, Germany). CD4+CD25− T (the responding cells) cells were positively isolated. The cells were washed and added to the assay culture system (see below).

2.5. Proliferation assay

Antigen-presenting cells were adjusted to 1.5×10^5 cells/ml and 100 ml were added to each well of a 96-well round bottom culture plate. The responding cells isolated from BALB/c mice were adjusted to 3.3×10^4 cells/ml and 100 ml were added to the antigen-presenting cells as described (Brines et al., 2001). The cells were cultured for 5 days and proliferation was measured by adding 0.5 µCi (Amersham, Buckinghamshire UK) to each well, incubating for 18 h and then harvesting the cells to count the incorporated thymidine.

2.6. Induction of colitis

Mice were anesthetized by chloral hydrate. DNBS (Sigma Chimica, Milan, Italy), 4 mg in 100 µl of 50% ethanol, was injected into the rectum through a catheter inserted 4.5 cm proximally to the anus. Carrier alone, 100 µl of 50% ethanol, was administered in control experiments. Thereafter, the animals were kept for 2 min in Trendelenburg position to avoid reflux. After 5 days, the animals were weighed and anesthetized with chloral hydrate, and the abdomen was opened by a midline incision. The colon was isolated, carefully cleaned from mesenterium, vessels and fat, rinsed with saline, and weighed. Thereafter, the colons were placed unstretched on a ruler, and a 5 cm segment of the distal colon was observed visually and for mean area of necrosis. The colons were then processed for histology and sero immunological analyses.

2.7. Experimental treatment

The animals were randomly divided into 7 groups: treatment started 1 h after DNBS and was continued daily until day 4th after DNBS

Treatment (*N*)	DNBS	Dosing
Group A = sham–vehicle (19)	No	0.1 ml i.p.
Group B = control–vehicle (19)	Yes	0.1 ml i.p
Group C = VGX-1027 0.1 mg/mouse (12)	Yes	0.1 ml i.p
Group D = VGX-1027 0.25 mg/mouse (12)	Yes	0.1 ml i.p
Group E = VGX-1027 0.5 mg/mouse (19)	Yes	0.1 ml i.p.
Group F = VGX-1027 2.5 mg/mouse (12)	Yes	0.2 ml p.o.
Group G = dexamethasone 1 mg/kg (19)	Yes	0.1 ml i.p

VGX-1027 was purchased from VGX Pharmaceuticals (Blue Bell, PA), dexamethasone was purchased from a local pharmacy. The doses of VGX-1027 used in this study were selected on the basis of previous *in vivo* studies (Stosic-Grujicic et al., 2007; Stojanovic et al., 2007). This dose of dexamethasone was chosen as the positive control drug on the basis of previous data indicating the capacity of this drug to ameliorate the course of a similar form of immunoinflammatory colitis (trinitrobenzene-induced colitis) in mice (Daniel et al., 2007)

2.8. Body weight variations

For the body weight variations, the mice were weighed on the day of colitis induction and then at the end of the experiment on day 5 post-DNBS challenge.

2.9. Assessment of diarrhoea and bleeding

The stool consistence was recorded daily with the following score system as described elsewhere (Daniel et al., 2007): 0=normally formed pellets; 2=pasty and partially formed lets; 4=liquid pellet. Bleeding: 0=no blood in hemoccult; 2=positive hemoccult; 4=gross bleeding from the rectum. The scores were added together resulting in a total clinical score ranging from 0 (healthy) to 8 (maximal activity of colitis).

2.10. Mucosal damage assessment

After weighing, the isolated distal colon was longitudinally opened by button-scissors and then pinned down on a cork-table with the mucosal surface oriented to the observer.

Mean necrosis area was measured macroscopically in mm^2/mouse.

Macroscopic damage score was scored on a 0 to 6 scale as described elsewhere (Palmen et al., 1998): 0=no damage; 1=localised hyperaemia and/or oedema; 2=linear ulcer<half of the width of the colon; 3=linear ulcer>half the width of the colon; 4=circular ulcer<1 cm; 5=circular ulcer between 1 and 2 cm; 6=circular ulcer>2 cm.

2.11. Histological scores of colitis

After fixation for 1 week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Thereafter, 7 mm sections were deparaffinised with xylene, stained with hematoxylin–eosin and trichromic van Giesson's stain, and observed in a Dialux 22 Leitz microscope (Leitz, Wetziar, Germany). In order to have a quantitative estimation of colon damage, sections ($N=6$ for each animal) were scored by two independent observers blinded to the experimental protocol as described elsewhere (Cuzzocrea et al., 2007). The following morphological criteria were considered: score 0=no damage; score 1=mild damage (focal epithelial oedema and necrosis); score 2=moderate damage (diffuse swelling and necrosis of the villi); score 3=severe damage (necrosis with presence of neutrophil infiltrate in the submucosa); score 4=highly severe damage (widespread necrosis with massive infiltrate with polymorphonuclear cells (PMN) and haemorrhage).

2.12. Myeloperoxidase (MPO) activity and determination of local cytokine levels

MPO activity, an indicator of PMN accumulation, was determined as previously described (Wallace, 1987). Five days after intracolonic injection of DNBS, the colon was removed, weighed, homogenised in 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7), and centrifuged for 30 min at 20,000 g at 41 °C. An aliquot of the supernatant was then allowed to react with 1.6 mM tetra-methyl-benzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 mmol of peroxide per min at 37 °C and expressed in units per gram weight of wet tissue. MPO activity was measured using 1.5 cm of the distal colon from each mouse.

2.13. Cytokines

Five cm of distal colon was homogenised in ice cold tissue protein extraction reagent (Pierce, Rockford, USA) containing phenylmethylsulfonyl fluoride, aprotinin, leupeptin, *N-p*-tosyl-L-lysine chloromethyl ketone, and L-1-tosylamiido-2-phenylethyl chloromethyl ketone, each at a concentration of 10 go/ml. The homogenate was then centrifuged at 18,000 g for 20 min at 4 °C. The supernatant was stored at −80 °C until use. Then, TNF-α, IL-1β, IFN-γ, IL-12p70 and IL-10 were quantified using enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Total protein concentration was assayed using the Bradford method (Bio-Rad, Hercules, CA). Data are shown as pg of cytokine per mg of protein.

2.14. Measurement of nuclear NF-κB/p65 in colons

NF-κB p65 translocation into the nucleus, as an index of NF-κB activation, was measured using a sandwich ELISA (Activ ELISATM; Imgenex, Analitica De Mori, Milano, Italy) according to the manufacturer's protocol. In brief, 1 g of colon from each of 5 mice out of a total of 35 mice for each experimental group was cut into small pieces, washed with cold PBS, homogenised in hypotonic buffer and centrifuged for 10 min at 10,000 rpm. The supernatant was used as cytoplasmic extract. Nuclear lysis buffer, 500 µl, was added to the pellet and centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was used as nuclear extract. The

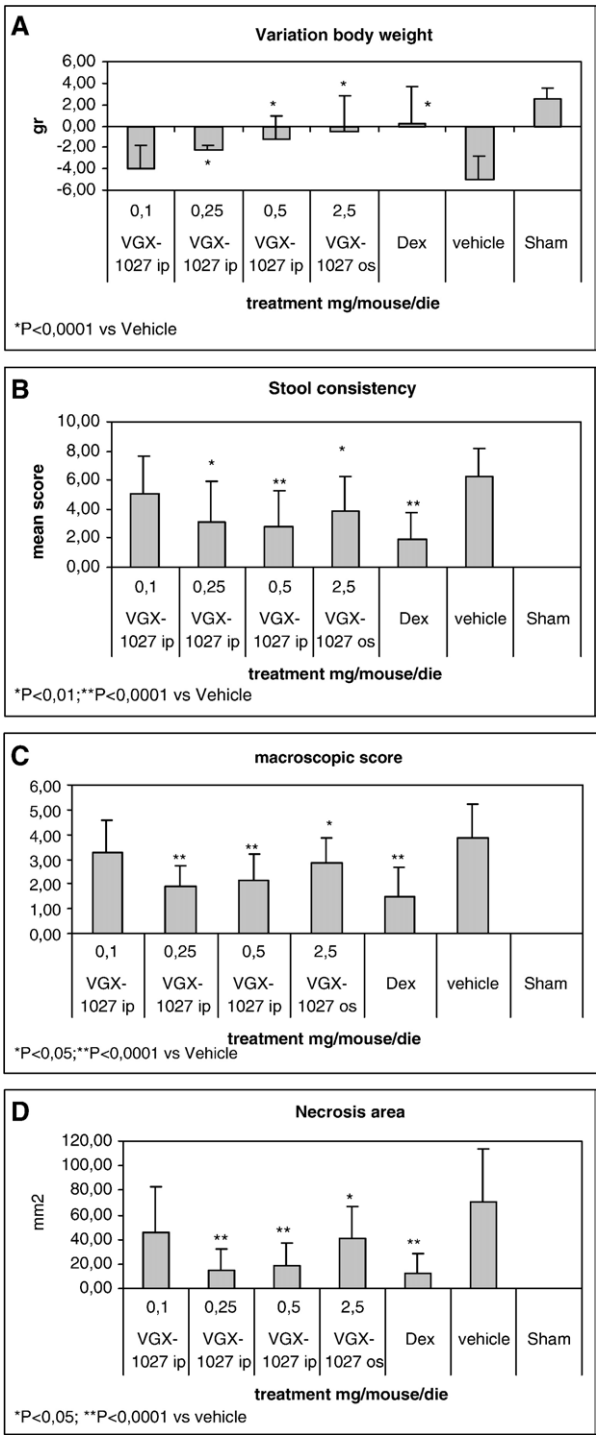

Fig. 3. Effects of VGX-1027 on clinical parameters in mice with DNBS-induced colitis. A Body weight variation on day 5; B stool consistency evaluation on day 5; C macroscopic score of colitis; D colon necrosis areas determined on day 5 post-DNBS challenge. Dex: dexamethasone 1 mg/kg.

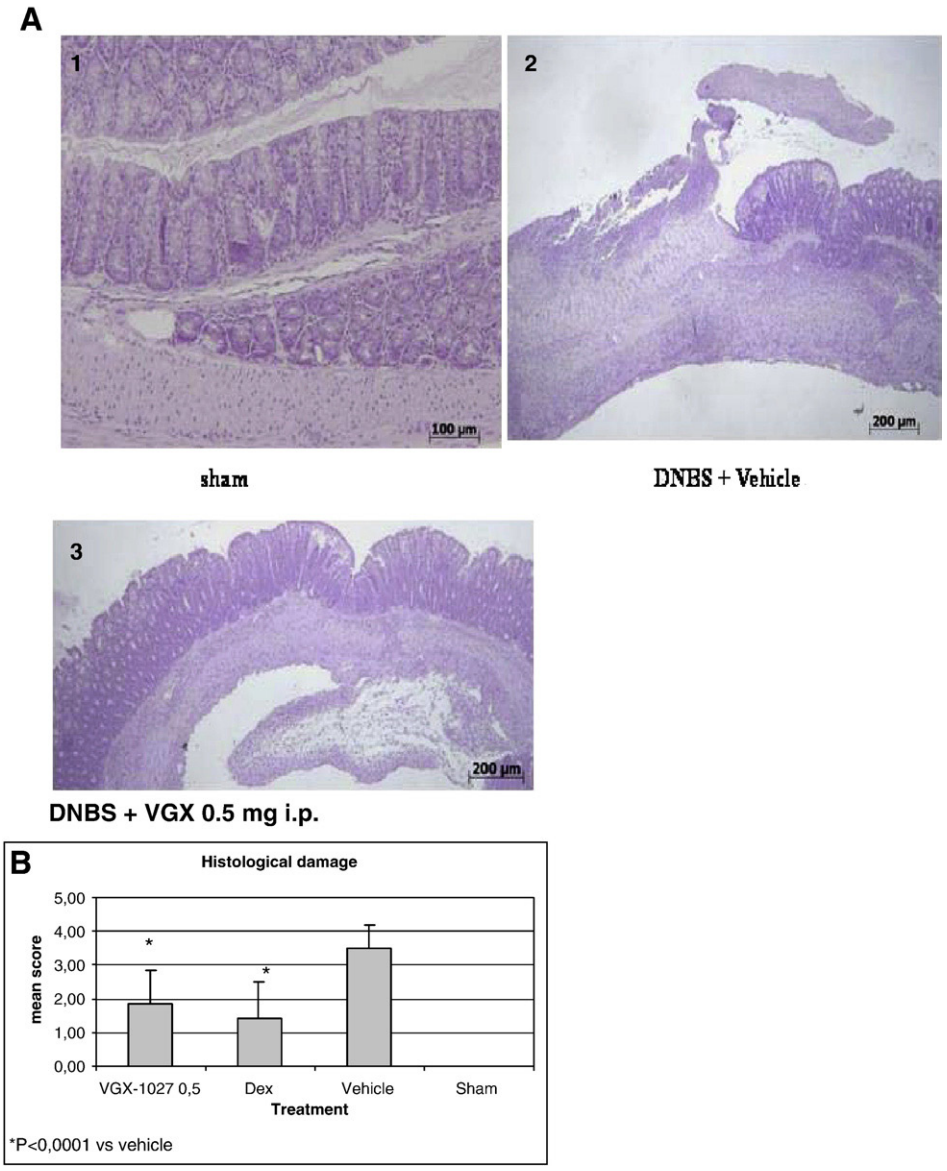

Fig. 4. Microscopic analysis of colon from mice with acute DNBS colitis. Animals were treated with 0.5 mg of VGX-1027 from 1 h after the induction of DNBS colitis until day 4 after DNBS administration. A, Photomicrograph of colon section after treatment with 50% ethanol (1), DNBS in 50% ethanol (2), DNBS plus 0.5 mg of VGX-1027; B, histologic score. Results are the mean ± S.D. from 12 mice per group.

anti-NF-κB p65 antibody-coated plate captured the nuclear or cytoplasmic NF-κB p65 of the samples (0.5 to 1 mg/ml of protein) and the amount of bound NF-κB p65 was detected by adding a secondary antibody followed by an alkaline phosphatase-conjugated antibody. The absorbance value for each well was determined at 405 nm using a microplate reader (Bio-Rad,

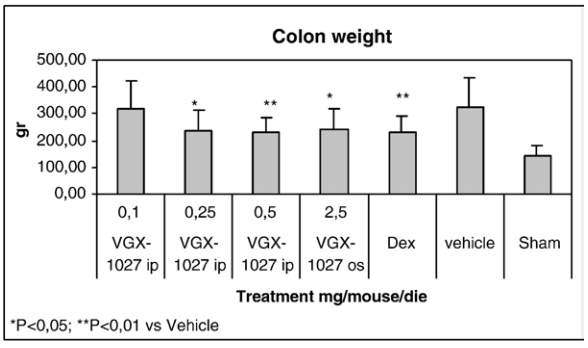

Fig. 5. Effect of VGX-1027 on the weight of 5 cm colon portions in mice with DNBS-induced colitis.

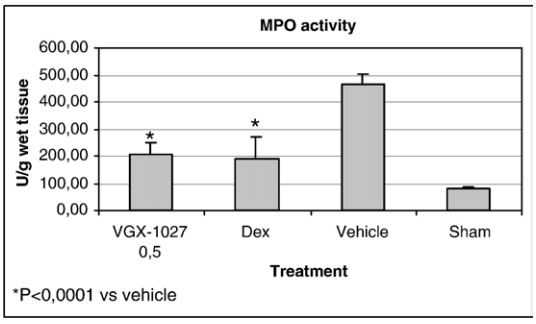

Fig. 6. MPO activity of colon from mice with acute DNBS-induced colitis. Animals were treated with 0.5 mg of VGX-1027 from 1 h after DNBS-challenge until day 4 after DNBS administration.

Hercules, CA, USA). The relative ratio of nuclear to cytoplasmic NF-κB p65 was calculated from the respective absorbance values.

2.15. Statistical analysis

Data are shown as mean values ± S.D. Statistical analysis was performed by Student's *t* test. A *P* value lower than 0.05 was taken as significant. Three to four independent experiments were performed for each group of mice. Clinical analyses (body weight variations and assessment of diarrhoea), the weight of the colons and the macroscopic observation of the colons were carried out in each mouse from the different groups. Histological data were assessed in 12 mice from groups A, B, E and G. The MPO contents of the colons and the local production of cytokines were measured in 7 mice from these same groups.

3. Results

3.1. VGX-1027 inhibits in vitro proliferation of CD4+CD25− Tcells

To study the effect of VGX-1027 on the proliferative response of CD4+CD25− T cells to enterobacteria, responding cells/antigen-presenting cells were co-cultured with either 1 or 10 μg/ml of VGX-1027. As shown in Fig. 2, 10 μg/ml, but not 1 μg/ml of VGX-1027 inhibited proliferation of responding cells.

3.2. Prevention of clinical and histological features of DNBS-induced colitis by VGX-1027

As expected, on day 5 after challenge with DNBS, the control mice treated with the vehicle of VGX-1027 exhibited clinical characteristics of colitis that included body weight loss and diarrhoea (Fig. 3A–B). These clinical features were associated with macroscopic changes characteristic of colitis with hyperaemia and inflammation (Fig. 3C) accompanied by large necrotic areas (Fig. 3D). Histological evaluation of the colons confirmed the macroscopic observations with transmural inflammation primarily composed of PMN and lymphocytes as well as loss of globule cells and widely spread fibrosis of the colon (Fig. 4A–B). There was also a marked increase in the weights of the colons of the control mice (Fig. 5). This represents an indirect marker of colitis as it is secondary to disease-associated intestinal wall thickening that correlates with intensity and severity of inflammation. We also observed high MPO activity in the inflamed colons of this group (Fig. 6). In contrast to these findings, the mice treated with VGX-1027 i.p. exhibited a dose-dependent amelioration of the clinical, histological

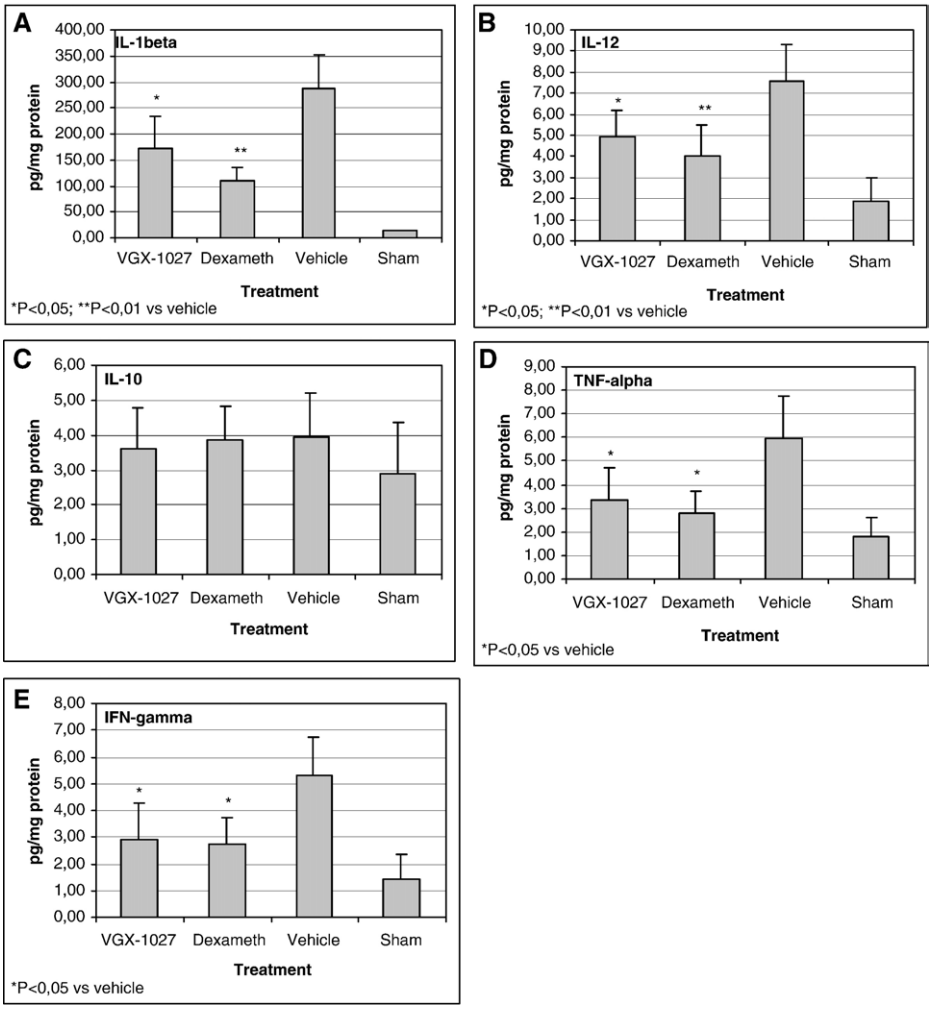

Fig. 7. Levels of cytokines in colon homogenates after treatment with 0.5 mg of VGX-1027 or 1 mg/kg of dexamethasone. Colonic specimens were taken on day 5 after DNBS challenge. Data are from 7 mice per group.

and laboratory readouts. Whilst a trend toward improvement of the necrosis area could be noticed in mice treated with the lowest dose of VGX-1027, all turned out to be markedly reduced in mice given the medium and, in particular, the highest dose of VGX-1027. Inhibition of colitis development of a similar efficacy to that achieved with the highest dose of the compound i.p. was also observed after p.o. treatment with 2.5 mg/mouse of VGX-1027. Hence, mice treated with either high dose VGX-1027 i.p. or with 2.5 mg/mouse of VGX-1027 p.o. exhibited significantly preservation of body weight loss, less severe diarrhoea, reduced necrotic areas of the colons, milder histological signs of colitis, lower increase of the weight of the colons and diminished MPO activity in the colons than vehicle-treated mice (Figs. 3–5). The degree of protection from these clinical and histological signs of colitis afforded by VGX-1027 was comparable to that afforded by 1 mg/kg of dexamethasone that was used as positive control drug (Figs. 3–5).

3.3. VGX-1027 inhibits production of proinflammatory cytokines in the colon during development of DNBS-induced colitis

Relative to vehicle-treated control mice, the treatment with VGX-1027 significantly reduced the contents of IL-1β, IL-12, TNF-α and IFNγ letting unaltered the values of IL-10 (Fig. 7). Similar findings were observed in the group of mice treated with the positive control drug dexamethasone.

3.4. VGX-1027 reduces colonic content of NF-κB p65

As shown in Fig. 8, increased levels of NF-κB p65 were found in tissue homogenates from vehicle-treated control mice with consequential increase of the nuclear/cytoplasmic ratio of the transcription factor subunit. In contrast, the nuclear values and the nuclear/cytoplasmic ratio of NF-κB p65 were significantly reduced both in the groups of mice treated with VGX-1027 and those treated with dexamethasone (Fig. 8).

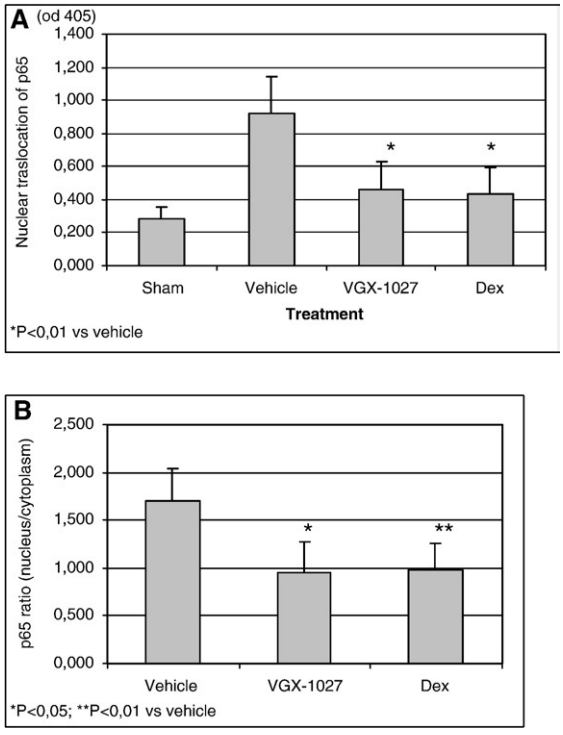

Fig. 8. A Nuclear translocation of NF-κB p65 in homogenate colon. B The relative ratio of nuclear to cytoplasmic p65.

4. Discussion

The data presented herein demonstrate that VGX-1027 markedly reduces proliferation of CD4+CD25− T cells to antigen-presenting cells loaded with enterobacterial extracts in an *in vitro* assay and powerfully suppressed the development of clinical, histological and immunological signs of DNBS-induced colitis in CD1 mice.

The *in vitro* assay of CD4+CD25− T cell proliferation in response to enterobacterial antigen-loaded, antigen-presenting cells, may resemble the aetiopathogenic events leading to development of the inflammatory bowel disease in humans. In fact, the intestine harbours a complex microflora composed of a large variety of indigenous aerobic and anaerobic bacteria that reside in close vicinity of intestinal lymphocytes without provoking their reactivity (Savage, 1998). The immunological mechanisms regulating the interaction between the mucosal immune system and the intestinal microflora and determining what allows the mucosal immune system to mount a rapid and effective response against pathogenic bacteria, viruses and parasites whilst remaining tolerant to the resident enteric microflora and dietary antigens are still unclear. However, several lines of evidence suggest that the inflammatory bowel disease may result as a breakdown of tolerance towards the normal enteric microflora (MacDonald et al., 2000; Duchmann et al., 1995). Reactivity against enteric bacteria has also been demonstrated in several animal models, including the severe combined immunodeficiency (SCID)-transfer model of colitis (Cong et al., 1998; Hans et al., 2000; Brines et al., 2001).

That inappropriate reactivity against enteric bacteria may also be involved in the pathogenesis of human inflammatory bowel disease is consistent with the observation that whilst lamina propria mononuclear cells of healthy individuals do not respond to autologous faecal extracts, this response occurs in the cells obtained from patients with inflammatory bowel disease (Duchmann et al., 1995).

In particular, recent studies in mice have shown that the majority of the enteroantigen-specific CD4+CD25− T cells reside both in the thymus and the periphery and that they are not dependent on previous antigen experience and express a mixed Th1/Th2 cytokine secretory capacity upon enteroantigenic stimulation (Gad et al., 2006). These cells also express a CD45RB-high, CD62L-high and CD44-low phenotype and represent a polyclonal population (Gad et al., 2006). It is thought that at homeostatic conditions CD25+ regulatory T cells can maintain peripheral tolerance on this CD4+CD25− T cell subset (Gad et al., 2006; Groux and Powrie, 1999). The beneficial effect of VGX-1027 in this assay is therefore consistent with the possibility that this compound has modulated the reactivity of intestinal immune cells toward enteric bacteria. Although we have not studied whether the inhibition of proliferation of CD4+CD25− T cells is secondary to an action of the test compound on the T cells or the antigen-

presenting cells, our previous study indicating that VGX-1027 target macrophages but not T cells (Stojanovic et al., 2007) is consistent with the hypothesis that the reduced proliferation is secondary to an effect of VGX-1027 on the function of antigen-presenting cells in this assay. Since the antigen-presenting cells were irradiated after the initial 18 h culture in the presence of VGX-1027 and the enterobacterial antigens, the putative action on these cells from VGX-1027 might have occurred in the early stages of antigen uptake and processing from the antigen-presenting cells. This may have in turned reduced the full efficiency of the so-treated antigen-presenting cells to let the enteroantigen-specific CD4+CD25− T cells subsequently added to the cultures to proliferate in response to enterobacterial-loaded antigen-presenting cells. The exact mechanism responsible for the inhibition of CD4+CD25− T cell proliferation from VGX-1027-pretreated antigen-presenting cells remains to be established. However, our previous data suggest that an inhibitory action of the test compound on the production of cytokines of the innate immune system that may favour CD4+T cell proliferation and expansion such as TNF-α and IL-1β has likely been involved.

These *in vitro* findings are strengthened by studies carried out in DNBS-induced inflammatory colitis that serves as a preclinical model of human inflammatory bowel diseases (reviewed by Hoffmann, 2002–2003). In this model, a single intracolonic instillation of DNBS provokes an acute inflammatory response of the intestine that can lead to widespread necrosis with massive infiltration of PMN and lymphocytes, bloody diarrhoea and weight loss.

The clinical and histological signs mentioned above were all successfully counteracted in a dose-dependent fashion by the i.p. treatment with VGX-1027. A golden p.o. dose of the compound previously shown to be effective in a mouse model of diabetes (Stosic-Grujicic et al., 2007) was effective in a manner comparable to the i.p. high dose-treatment. We have also shown that the development of DNBS-induced colitis is associated with local production of proinflammatory cytokines of both innate and adaptive immunity such as IL-1β, IL-12, TNF-α and IFNγ and augmented the nuclear contents of the NF-κB p65 and increased the nuclear/cytoplasmic ratio of this transcription factor subunit. In agreement with the histological protection afforded by VGX-1027 we have also observed that the so-treated animals also exhibited significantly reduced colonic production of these cytokines and reduced NF-κB activation as compared to vehicle-treated controls. It should however be noticed that these data do not allow us to discriminate whether this phenomenon reflects a primary action of VGX-1027 on NF-κB signalling pathways and the cytokine secretory capacity of inflammatory cells or whether it is secondary to the reduced number of infiltrating mononuclear cells and PMN in the colons of VGX-1027-treated mice. However, the selective capacity of VGX-1027 to influence production of TNF-α and activation of NF-κB and p38 MAP kinases (Stojanovic et al., 2007) suggests that down-regulation from these cells of TNF-α and possibly IL-1β production may have been important in determining the lower migration of inflammatory cells to the colons and the secondary local production of these and other proinflammatory cytokines. This hypothesis is also consistent with the presently observed reduction of IFN-γ levels in the colons of VGX-1027-treated mice in spite of our observation that VGX-1027 spares murine CD4+ T cell function *in vitro* as shown by its inability to reduce proliferation or cytokine (IFN-γ, IL-4) synthesis induced by either Concanavalin A or anti-CD3 and anti-CD28 monoclonal antibody (Stojanovic et al., 2007).

It also emerges from the local measurement of cytokines in the colons of DNBS-challenged mice that the levels of IL-10 were lower than those of the other cytokines studied and that VGX-1027 had no influence on these levels. The relatively reduced production of IL-10 in the colons may be associated with the development of DNBS-induced colitis as endogenous IL-10 plays a protective role in the pathogenesis of inflammatory bowel diseases (Leach et al., 1999; Li and He, 2004; Gordon et al., 2005) and because IL-10 knock out mice suffer from spontaneous development of colitis (Davidson et al., 2000). On the other hand, the similar levels of IL-10 found in the colons of VGX-1027-treated and vehicle-treated mice indicate that the previously reported capacity of VGX-1027 to reduce LPS-induced IL-10 secretion from murine peritoneal cells (Stojanovic et al., 2007) has not occurred in this experimental setting. These discordant findings may be secondary to different experimental conditions as well as to the possibility that local colonic contents of cytokines in DNBS-challenged mice treated with VGX-1027 may not directly mirror the effect of the compound on the secretion of the cytokine.

It is also worthy noticing that the clinical, histological and immunological effects of VGX-1027 in DNBS-challenged mice were of a similar magnitude to that afforded by dexamethasone used to treat patients with inflammatory bowel diseases (Irving et al., 2007). This further indicates that VGX-1027 possesses an immunopharmacological profile that warrants further considerations for its potential use in the treatment of human inflammatory bowel diseases.

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