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The new low calcemic vitamin D analog 22-ene-25-oxa-vitamin D prominently ameliorates Th1-mediated colitis in mice

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Vitamin D analog therapy of mouse colitis

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ABBREVIATIONS: BW, body weight; CD, Crohn´s Disease; DC, Dendritic cells; IBD, inflammatory bowel disease; LBD, ligand binding domain; MPO, myeloperoxidase; PBMCs, peripheral blood mononuclear cells; RXR, Retinoid X receptor; TNBS, Trinitrobenzene sulfonic acid; Th1, T helper cell type 1; Th2, T helper cell type 2; TNBS, Trinitrobenzene sulfonic acid; VDR, vitamin D receptor; VDRE, vitamin D responsive element;

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ABSTRACT

In addition to its well-defined role as key regulator of calcium and bone metabolism, 1,25-dihydroxyvitamin D_3 (calcitriol) has been established as a potent modulator of immune cell function. Still, due to the hypercalcemic toxicity occurring after systemic application of the parent compound its clinical application as an immunosuppressant has been hampered. Recently, we described 22-ene-25-oxa vitamin D (ZK156979) as a representative of a novel class of low calcemic vitamin D analogs with a well preserved immunosuppressive activity in vitro. Here, in vivo a colitis was induced by applying a rectal enema of TNBS to male Balb/c mice, and calcitriol [0.2 µg/kg] or ZK156979 [0.1 to 2.0 µg/kg] was given intraperitoneally from day 0-3 or 3-5. Body mass and clinical activity score of colitis were recorded daily. Colon tissue was analyzed macroscopically and microscopically, myeloperoxidase activity as well as cytokine levels (TNF-α, IFN-γ, IL-10, IL-4) were determined by ELISA, T-bet expression by immunoblot analysis. We found that treatment with ZK156979 clearly reduced the severity of TNBS-colitis without exhibiting calcemic effects. Both, early and late treatment abrogated body weight loss, diarrhea, and macroscopic intestinal inflammation with a potency comparable to calcitriol. The therapeutic effect of ZK156979 was accompanied by a down-regulation of MPO-activity, TNF-α-, IFN-γ– and T-bet expression, while local tissue IL-10 and IL-4 protein levels increased. To conclude, our data provide first clear evidence that ZK156979 exhibits a beneficial prophylactic as well as therapeutic profile in Th1-like experimental colitis offering new therapeutic options for the treatment of human inflammatory bowel diseases.

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INTRODUCTION

1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$, calcitriol), the active metabolite of vitamin D, is an important regulator of calcium homeostasis, bone development and metabolism. Additionally, several studies indicated its role in the functional modulation of the immune system generally attributed to its capacity to regulate cell growth and differentiation (Manolagas, et al., 1985). The Vitamin D receptor (VDR), a member of the superfamiliy of nuclear hormone receptors, has been shown to be present in monocytes, monocyte-derived cells and T- and B-lymphocytes (Bhalla, et al., 1983;Provvedini, et al., 1983;Veldman, et al., 2000). The binding of the ligand to the VDR induces a conformational change in the ligand binding domain with consequent promotion of heterodimerization with the retinoid X receptor (RXR) and dissociation of corepressors and association with coactivators (Dong, et al., 2003). Thus the VDR functions as a ligand–activated transcription factor that binds to specific vitamin D responsive elements (VDREs) in responsive genes (Dong, et al., 2003). While accepted as the major pathway this classical view of calcitriol action has been challenged by recent investigations describing numerous rapid, presumably non-transcriptional, effects (Norman, et al., 2004). Although many candidates had been discussed no other targets than the VDR have been confirmed so far (Mizwicki, et al., 2004). However, the recent finding of a new endosomal high affinity G-protein-coupled receptor for 17ß-estradiol, GPR30, may also accelerate the search for an alternative calcitriol target (Revankar, et al., 2005).

VDR ligands have pleiotropic activities in immune regulation. It is intriguing that several different molecular mechanisms of cytokine inhibition by calcitriol exist. Antigen presenting cells as well as T cells have been indicated to function as direct targets of calcitriol, leading to the inhibition of pathogenic effector T helper type 1 (Th1) cytokines as demonstrated in the prevention of Th1-mediated disease-models, while the Th2 compartment is not affected or even augmented and T cells with regulatory properties are also induced, mainly via the

promotion of tolerogenic dendritic cells (DCs) (Griffin, et al., 2003). To translate the immunosuppressive capacities of calcitriol into effective immunointervention great efforts have been put into the design of structural analogs of calcitriol that are devoid of adverse effects on calcium levels due to a reduced calcemic activity (Griffin, et al., 2003;Mathieu and Adorini, 2002;Steinmeyer, et al., 2000;Zugel, et al., 2002). The investigation of the 25-oxa series generated a large number of calcitriol analogs exhibiting substantial dissociation between possible immunomodulatory capacities and undesired hypercalcemia. Especially, the combination of the 22-ene situation with the 25-oxa element as mentioned for ZK156979 (Figure 1) yielded a very promising set of new analogs for further characterization in animal models resembling human autoimmune diseases (Steinmeyer, et al., 2000). Recently, we showed that one representative, 22-ene-25-oxa vitamin D (ZK156979), may qualify as a promising member of this novel class of vitamin D analogs as it revealed prominent immunomodulatory and suppressive characteristics in vitro in human peripheral blood mononuclear cells (PBMCs) with inhibition of Th1 cytokines whereas the Th2 compartment was augmented (Daniel, et al., 2005).

The pathogenesis of human Crohn´s disease (CD) remains poorly understood. The establishment of the pathophysiology may be the result of an uncontrolled or inadequate cellular immune response in the intestinal mucosa to hitherto unknown agents, probably constituents of the luminal content that inadequately pass through the epithelial barrier or become accessible together with an inflammatory costimulus (Brandtzaeg, et al., 1997). Recent data suggest that CD and a variety of other inflammatory diseases reflect an excessive Th1 response (Neurath, et al., 1995). Indeed, it has been demonstrated that agents inhibiting a Th1 response or promoting a Th2 profile are beneficial in improving disease symptoms (Glimcher and Murphy, 2000). Intestinal inflammation induced by intrarectal instillation of the hapten 2,4,6 trinitrobenzene sulfonic acid (TNBS) in mice resembles many of the clinical,

histopathologic, and immune characteristics of CD in humans including the progression to chronic colitis with a severe transmural inflammation (Strober, et al., 2002).

Herein, we will demonstrate that treatment with ZK156979 strongly reduced the severity of Th1-mediated TNBS-colitis. Without affecting calcium levels, but with potency comparable to the parent compound calcitriol, the analog abrogated body weight loss, and diarrhea, macroscopic and microscopic intestinal inflammation. Moreover, the therapeutic effects of ZK156979 were associated with a down-regulation of MPO-activity, IFN-γ, TNF-α and T-bet- expression, while IL-10 and IL-4 were distinctly induced. Thus this member of the novel low calcemic vitamin D analogs may hold the promise for a considerable therapeutic potential in T cell-mediated diseases including inflammatory bowel disease (IBD).

Methods

Animals

Male, 8-week old BALB/c mice weighing about 20 g were used in all experiments (Charles River Laboratories, Sulzfeld, Germany). All studies were performed under approval of the Ethics Committee of Darmstadt/Hessen (Germany, F134/03) and are in agreement with the guidelines for the proper use of animals in biomedical research. The mice were kept in polycarbonate cages in temperature-controlled rooms with a 12-h light/dark cycle and fed standard mouse chow and tap water. At the end of the experiments mice were sacrificed by cervical dislocation under isoflurane anesthesia (Forene, Abbott, Wiesbaden, Germany).

Induction of colitis by the haptenating agent TNBS

TNBS-colitis

T helper type 1-mediated colitis was induced via rectal instillation of the haptenating agent trinitrobenzene sulfonic acid (TNBS; 2,4,6-TNBS, Sigma-Aldrich, Deisenhofen, Germany) at a concentration of 2% in 45% ethanol and at a dose of 100 mg/kg body weight (BW) to mildly anaesthetized mice through a 3.5 F catheter carefully inserted into the rectum. The catheter tip was inserted 4 cm proximal to the anal verge. Mice were carefully held in a vertical position for 1 min following the TNBS-instillation procedure to ensure distribution of the TNBS within the entire colon and caecum. Control animals were administered 45% ethanol alone using the same technique.

Administration of calcitriol or ZK156979 and study design

Calcitriol was purchased from Biomol (Hamburg, Germany), dissolved in ethanol at a concentration of 1 x 10^{-2} mol/L and kept at –80°C until use. Calcitriol was administered i.p. at a dose of 0.2 µg/kg BW in 0.9% NaCl solution containing 0.085% Myrj53 (Sigma,

Deisenhofen, Germany), the solutions were prepared freshly every day. 22-ene-25-oxa vitamin D (ZK156979) was synthesized and supplied by the Department of Medical Chemistry at Schering AG (Berlin, Germany) and administered i.p. at a dose of 0.1 to 2.0 µg/kg BW in 0.9% NaCl solution containing 0.085% Myrj53 (Sigma, Deisenhofen, Germany), respectively. Two protocols were used: (A) *Acute colitis*: calcitriol or ZK156979 was administered i.p. 2 h before the instillation of the TNBS enema and during the following three days. On day three the colon was removed following cervical dislocation under isoflurane anesthesia. (B) *Established ongoing colitis*: calcitriol or ZK156979 was administered from day three to five following the instillation of the TNBS-enema. On day five the colon was removed for analysis.

Analytical procedures for determining serum calcium and creatinine

Serum calcium levels were determined after treatment with calcitriol or ZK156979 at the end of the respective experiments. The concentration was measured by the calcium-cresolphtalein colorimetric assay according to the manufacturer´s instructions (Hitado, Möhnsee Delecke, Germany). Serum creatinine levels were measured using the alkaline picrate method (Hitado, Möhnsee Delecke, Germany).

Assessment of inflammation and colitis severity

Clinical activity score of colitis

The BW, as well as the stool consistency and rectal bleeding were examined daily to assess the clinical severity of colitis. The determination of the clinical activity score of colitis was performed independently by two investigators being unaware of the treatment protocol using a scoring system described previously in detail (Hartmann, et al., 2000). In brief, the loss of BW was scored as follows: 0: no weight loss; 1: weight loss of 1-5%; 2: weight loss of 5-10%; 3: loss of 10-20% and 4: weight loss >20%. Assessment of diarrhea (stool consistency):

0: normally formed pellets; 2: pasty and semiformed pellets; 4: liquid stools. Bleeding: 0: no blood in hemoccult; 2: positive hemoccult; 4: gross bleeding from the rectum. The results of these scoring parameters were added resulting in a total clinical score ranging from 0 (healthy) to 12 (maximal ill/activity of colitis).

Colon weight and colon length

The length and weight of the colon were used as indirect markers of disease-associated intestinal wall thickening correlating with the intensity of inflammation.

Macroscopic scoring system

The macroscopic colonic damage was assessed using the scoring system of Wallace and Keenan (Wallace and Keenan, 1990) which takes into account the area of inflammation and the presence or absence of ulcers. The criteria for the evaluation of macroscopic damage were based on a semi-quantitative scoring system. Features were graded as follows: 0: no ulcer, no inflammation; 1: no ulcer, local hyperemia; 2: ulceration without hyperemia; 3: ulceration and inflammation at one site only; 4: two or more sites of ulceration and inflammation; 5: ulceration extending over more than 2 cm.

Histological analysis of the colon

For histological examination a sample of colonic tissue located precisely 3 cm above the anal canal was obtained from the mice of all treatment groups. The colonic tissues were fixed in 10% neutral buffered formalin and embedded in paraffin for histological analysis. 4 µm-sections were deparaffinized with xylene and stained with hematoxylin and eosin (H&E) using routine techniques. Tissues were graded semi-quantitatively from 0 to 5 in a blinded fashion according to previously described criteria (Asseman, et al., 1999). 0: no changes; 1: minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; 2: mild scattered to diffuse inflammatory cell infiltrates, sometimes extending

into the submucosa and associated erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; 3: mild to moderate inflammatory cell infiltrates that were sometimes transmural and associated with ulcers, with moderate epithelial hyperplasia and mucin depletion; 4: marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; 5: marked transmural inflammation with severe ulceration and loss of intestinal glands.

Measurement of myeloperoxidase activity

For determination of the neutrophil infiltration in the inflamed colon tissue the myeloperoxidase (MPO) activity assay according to the method described by Bradley at al. (Bradley, et al., 1982) with slight modifications was used. The enzyme activity was analyzed photometrically as the MPO-catalyzed change of absorbance at 650 nm occurring by the redox reaction of 3,3,5,5-tetramethylbenzidine dihydrochloride (TMB-substrate, Sigma-Aldrich, Deisenhofen, Germany). MPO (Sigma-Aldrich, Deisenhofen, Germany) was used as an internal standard. Values are expressed as MPO units per gram of wet tissue.

Colonic protein extraction

The respective colon segment was removed, washed in phosphate buffered saline to wash-off any fecal matter, snap-frozen in liquid nitrogen and stored at –80°C until use. The extraction of colonic protein was performed using the Active Motif Nuclear cell extraction kit according to the instructions (Active Motif Nuclear extract kit, Rixensart, Belgium). Briefly, the excised colon was washed with ice-cold PBS and homogenized in ice-cold complete lysis buffer. After an incubation step of 30 min on ice the lysates were centrifuged twice at 4000 x g at 4°C for 20 min. Aliquots of the resulting extracts were then analyzed for their protein content

using the BioRad colorimetric assay according to the method of Bradford (BioRad Laboratories, Muenchen, Germany) and stored at –80°C until use.

Cytokine assays

The amount of murine TNF-α, IFN-γ, IL-4, IL-10 in the colonic protein lysates were quantified by commercially available ELISA kits (R&D Systems, Abingdon, England) according to the manufacturer´s instructions and adapted to the protein content of the colon tissue probe.

Western immunoblot analysis

After addition of sample buffer to the colonic protein extracts and boiling samples at 95°C for 5 min 150 µg of total protein lysate were separated with a 10% SDS-polyacrylamide gel (T-bet). Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Kassel, Germany) and the membrane was blocked for 1 h at room temperature with 3% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). The level of proteins was assayed using the appropriate primary antibody (mouse T-bet sc-21749, from Santa Cruz Biotechnology, Santa Cruz, USA) overnight at +4°C. Immunoreactivity was demonstrated by an ECL-system (Amersham Pharmacia Biotech, Buckinghamshire, UK) using an appropriate HRP-conjugated secondary antibody (NA931, from Amersham Biosciences Europe, Freiburg, Germany). Bands were detected after exposure to Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were re-probed with an actin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). For quantitative analysis, the bands were detected with scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProViDoc software. (Desaga, Wiesloch, Germany).

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Statistical analysis

All data are expressed as means ± SEM. Statistical significance of differences between TNBS- and calcitriol- or ZK156979-treated was determined by the unpaired two-tailed Student´s t test (Sigma Stat, Chicago, IL). Differences were considered statistically significant with $p<0.05$.

Results

Blood calcium and creatinine profile

Serum calcium and creatinine levels in mice were analyzed on day three following the first application of calcitriol or 22-ene-25-oxa vitamin D (ZK156979). Mice treated with calcitriol showed a trend toward hypercalcemia relative to controls, in contrast however the application of ZK156979 did not cause any significant changes in calcium levels (Table 1).

Impact of ZK156979 compared to calcitriol on inflammation and colitis severity in acute and established ongoing Th1-mediated TNBS-colitis

We first investigated the effect of the vitamin D analog ZK156979 on the prevention of the acute TNBS-colitis (Th1-model). In this acute model, the first dose of ZK156979 or calcitriol was administered i.p. two hours before the instillation of the TNBS-enema and subsequently during the following three days. On day three the colon was removed for analysis. Mice treated with TNBS in 45% ethanol developed severe diarrhea accompanied by an extensive wasting disease. As demonstrated in Figures 2-7, the application of ZK156979 resulted in a remarkable amelioration of the wasting disease compared with TNBS-treated mice, as assessed by animal weight loss, as well as clinical, macroscopic, microscopic and immunological parameters of colitis.

To evaluate a dose-response profile of the in vivo therapeutic potential of ZK156979 compared to the mother compound calcitriol in this Th1-mediated colitis model a dose range from 0.1 to 2.0 µg/kg was administered i.p. to male Balb/c mice. Dose-response data for ZK156979 were established following assessment of inflammation, colitis severity and macroscopic colitis parameters and are shown in Figure 2*A-D*. After TNBS instillation a fast decrease in BW was observed as a result of colitis. Control mice treated with 45% ethanol alone failed to develop wasting disease and revealed a healthy appearance. In contrast, mice

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treated once daily with ZK156979 i.p.on day three exhibited a significant reduction of the colitis-associated weight loss correlating with the dose of the analog applied [0.1 to 2.0 µg/kg] (Figure 2*A*).

Clinical activity score of colitis was analyzed as described in detail in the Materials and Methods section. Colitis was significantly and dose-dependently blunted in the ZK156979-treated mice (Figure 2*B*). The TNBS enema led to a significant reduction of mean colon length, as assessed on day three combined with a prominent thickening of the colon wall resulting in a significant increase of the weights of 6-cm portions of distal colons. ZK156979 i.p. reduced the extent of the TNBS-associated colon shortening as well as the colitis-mediated increase of colon weight significantly in a dose-dependent fashion ($p<0.001$ vs. TNBS-group, Figure 2*C+D*).

Macroscopic analysis of colons obtained three days after colitis initiation by TNBS demonstrated a striking hyperemia, necrosis, and inflammation compared with ethanol-treated control groups that showed only faint signs of inflammation (Figure 3*A+B*). To determine macroscopic changes quantitatively the severity of colonic inflammation and ulceration was graded using criteria for macroscopic scoring as described in detail in the Materials and Methods section. The administration of the vitamin D analogue ZK156979 significantly and dose-dependently improved macroscopic scores on day three after TNBS instillation, with the colons showing a massive reduction of colitis-associated hyperemia and inflammation, when the 2.0 µg/kg dose of ZK156979 was used (Figure 3*A+B*).

The severity of colonic inflammation and ulceration was further evaluated by histological examinations (Figure 3*C*). By day 3 transmural inflammation, characterized by infiltration of inflammatory cells, predominantly neutrophils and lymphocytes, was accompanied by ulcerations, loss of globlet cells and fibrosis found throughout the colon. Treatment with ZK156979 caused a dose-dependent improvement of these symptoms, leading to restoration of the histological appearance of the mucosa and submucosa compared with the TNBS group

and the ethanol-treated control group. These histological findings were graded using criteria for histological grading of colitis. ZK156979 also improved the histological score three days after TNBS instillation significantly (Figure 3*D*).

In a next set of experiments we evaluated, whether ZK156979 in addition to its capacities to prevent acute TNBS colitis, might also be able to reverse established TNBS colitis. To this end, the first dose of ZK156979 or calcitriol was administered on the third day after disease initiation with the TNBS enema and continued subsequently for the next two days. The colon was then removed on day five after the TNBS instillation. With similar potencies compared to calcitriol ZK156979 treatment also blocked the development of the disease and led to an improvement as indicated by a regain of lost BW, reduction of clinical activity score, as well as by a significant amelioration of macroscopic signs of colitis (Figures 4*A-H*). This data indicate that ZK156979 is effective not only as an experimental preventive drug but also as a true therapeutic for established colitis.

Impact of ZK156979 in comparison to calcitriol on the inflammatory response in acute TNBS colitis

Because in vitro we recently could demonstrate substantial anti-inflammatory capacities of ZK156979, we investigated in this study, whether ZK156979 could affect the local production of selected inflammatory mediators in mice with acute TNBS colitis in vivo. First, to quantify neutrophil infiltration during ongoing disease we evaluated the effect of ZK156979 on MPO activity in colon extracts three days after the instillation of the TNBS enema. In the acute phase of colitis (day three), colonic MPO-activity values were significantly increased in comparison with the ethanol-treated control group (TNBS group: 11.5±0.8 vs. control-group: 0.25±0.2; Figure 5*C*). In contrast, MPO activities in animals treated with ZK156979 reached levels 25% (0.2 µg/kg) and 65% (2.0 µg/kg) below the TNBS group.

In the initial stages of TNBS-induced colitis key inflammatory cytokines such as TNF-α and IFN-γ are secreted. Therefore we tested, whether the vitamin D analog is also able to regulate the production of pro-inflammatory cytokines in vivo. The administration of ZK156979 led to a significant down regulation of TNF-α and IFN-γ protein expression (Figure 5*A*+*B*). The observed down regulation of the mediators of a Th1 immune response after treatment with ZK156979 was further confirmed by Western blot analysis of the Th1-relevant transcription factor T-bet, which was rapidly and specifically induced in Th1 differentiated lymphocytes (Figure 6*A*+*B*).

Next we analyzed the in vivo impact of ZK156979 on the promotion of a Th2 profile, which was indicated by our in vitro studies before. Both, the parent compound calcitriol and ZK156979 markedly up-regulated IL-4 in both doses analysed, while not affecting calcium levels. Moreover, calcitriol and ZK156979 both significantly enhanced the production of the anti-inflammatory cytokine IL-10 in comparison the untreated TNBS group (Figure 7*A*+*B*).

Discussion

The present investigation performed in acute and established Th1 colitis clearly demonstrates the potent immunomodulatory capacity of the new and low calcemic analog 22-ene-25-oxa vitamin D ZK156979. These data confirm our recent in vitro findings with this analog in PHA stimulated human PBMCs (Daniel, et al., 2005). The substantial amelioration of clinical parameters of Th1-mediated TNBS colitis was accompanied by a significant down regulation of the inflammatory response as assessed by MPO activity and local TNF-α and IFN-γ levels. The distinct inhibition of the Th1-like immune response in this colitis model following treatment with ZK156979 was further supported by demonstrating that the Th1-prototypic transcription factor T-bet was reduced significantly. Remarkably, ZK156979 simultaneously promoted a Th2 profile as indicated by an induction of IL-4, and further substantiated by a significant increase of the anti-inflammatory cytokine IL-10.

Several epidemiologic studies stressed the impact of a vitamin D deficiency not only as a risk factor for skeletal disorders but also for infectious, malignant and autoimmune diseases such as IBD, rheumatoid arthritis, multiple sclerosis and type 1 diabetes (Cantorna, et al., 2004;Hypponen, et al., 2001;Mahon, et al., 2003;Peterlik and Cross, 2005;Zella and DeLuca, 2003). Reduced vitamin D levels have been identified as a major problem especially among patients with IBD, even when the disease is in remission (Froicu, et al., 2003). Using animal models the pleiotropic pathophysologic consequences of insufficient calcitriol supplementation or genetic VDR defects have been underlined. In two different experimental models of colitis VDR deficiency caused severe inflammatory injury of the gastrointestinal tract. To extend this hypothesis one step further, the VDR gene maps to a region on chromosome 12 that has been linked to IBD by genome screening techniques. Analysis of single nucleotide polymorphisms in VDR characterized in patients with CD provided preliminary evidence for a genetic association of susceptibility to CD and these VDR variants

mapping to one of the candidate regions determined by linkage analysis (Simmons, et al., 2000).

The plethora of actions of calcitriol in various systems hold the promise for a wide spectrum of clinical applications of VDR ligands in treatments of inflammatory disorders including rheumatoid arthritis, psoriasis, multiple sclerosis as well as IBD (DeLuca and Cantorna, 2001;Griffin, et al., 2003;Mahon, et al., 2003). However, the clinical application of calcitriol has been hampered severely due to its hypercalcemic side effects and soft tissue calcification. To avoid these adverse effects on bone and calcium metabolism a great effort has been put into the design of structural analogs of calcitriol with reduced hypercalcemic profile (Verstuyf, et al., 2000). The mechanistic basis for this reduced hypercalcemic potencies has not been fully understood, but apparently and in addition to the introduction of modifications that favor high intracellular accumulation, such analogs may induce conformational changes of the VDR that alter the structural and functional properties of the entire DNA binding complex (Verlinden, et al., 2001). Therefore, it has been proposed that analogs of calcitriol, when bound to VDR, may induce modified gene expression profiles compared to the physiologic calcitriol/VDR complex (Carlberg, 2003;Verlinden, et al., 2001). Further characterization of the molecular events underlying the separation of hypercalcemic and non-hypercalcemic effects of calcitriol agonists may allow the development of even more selective analogs e.g. as immunosuppressive agents. As pointed out previously ZK156979 is characterized by an altered side chain consisting of a 22,23-double bond and a 25-oxa-modification. These modifications result in a minute reduction of receptor affinity and a 100-fold lower hypercalcemic activity compared to the parent compound calcitriol (Steinmeyer, et al., 2000).

The central step in calcitriol signaling is the conformational change of VDR´s ligand binding domain (LBD) and the resulting exchange of protein-protein interaction partners. DNA-bound

VDR-RXR heterodimers have been indicated to function as the molecular switches in calcitriol signaling. Thereby, the functional profile of VDR ligands might be best explained by the agonistic, antagonistic and non-agonistic conformation of the VDR´s LBD within this molecular switch. The positioning of helix 12 clearly plays a central role in this aspect, because changes here resulted in different interactions with either corepressor or coactivator proteins (Carlberg, 2003;Tocchini-Valentini, et al., 2004). Most of the recently developed calcitriol derivatives function as agonists, which also holds true for ZK156979 used here.

Inhibition of factors leading to Th1 polarization following activation of innate responses is likely to play a pivotal role for the avoidance of autoimmunity (Szabo, et al., 2003). A number of recent studies have clearly indicated a strong impact of calcitriol in the negative regulation of the Th1-type immunity (DeLuca and Cantorna, 2001;Griffin, et al., 2003). The pro-inflammatory activities of Th1 immunity are associated with a destructive lymphocytic tissue infiltration that is locally driven by DCs presenting antigen and concomitantly providing the cytokine IL-12 that further enhances both IFN-γ secretion by effector Th1 lymphocytes and differentiation of additional Th1 cells from naïve precursors . In addition to calcitriol augmented reduction of the inflammatory side, it is important to note that calcitriol also leads to an induction of the anti-inflammatory cytokine IL-10 that may be derived from monocytes, DC and a subtype of regulatory T cells (Barrat, et al., 2002;Boonstra, et al., 2001;Griffin, et al., 2003). While IL-10 directly down modulates Th1 activity, its impact on T helper polarization seems to be more sustained by promoting the development of a Th2 phenotype. These IL-10 effects are supposed to operate through an induction of IL-4, although this was not confirmed in all studies (Boonstra, et al., 2001;Staeva-Vieira and Freedman, 2002;Pichler, et al., 2002).

In a previous in vitro study, using human PBMCs, we clearly demonstrated prominent immunosuppressive capacities for ZK156979 resulting in a strong inhibition of the PHA-

induced Th1-response (IFN-γ and TNF-α), as well as of the macrophage-product IL-1β with a molar potency on cytokine expression being only marginally reduced compared to calcitriol (Daniel, et al., 2005). Additionally, in these in vitro experiments ZK156979 in accordance with calcitriol also affected the Th2 response, leading to a significant increase of IL-4 and IL-10. The latter observations are consistent with the present findings provided in this study demonstrating that the immunosuppressive capacities of ZK156979 are transferable into the in vivo model of Th1-mediated TNBS colitis. In the early, acute phase of bowel inflammation, there is an overlap of the innate and acquired immune responses, with multiple mediator pathways involved, such as chemokines and cytokines. TNBS-induced colitis exhibits clinical, histological, and microscopic similarities to CD, and the course of colonic injury has been well characterized (Strober, et al., 2002). ZK156979 strongly and dose-dependently reduced the inflammatory response by down-regulating the production of different mediators implicated in local and systemic damage as marker for neutrophil infiltration and pro-inflammatory cytokines. However, in contrast to calcitriol even when a 10-fold higher concentration of ZK156979 was applied, ZK156979 had no impact on serum calcium levels. The balance of the Th1/Th2-type cytokines has a definite function for the establishment of a chronic disease. Th1 lymphokines, if not counter-balanced by either Th2 or regulatory cytokines, possess potent pro-inflammatory features leading to destructive tissue injury (Neurath, et al., 2002).

In this study, we show, that treatment with the low calcemic vitamin D analog ZK156979 strongly down-regulated the Th1 cytokine profile, decreasing TNF-α, IFN-γ production and T-bet expression, while on the other hand it led to an up-regulation of IL-4 and IL-10.

To conclude, VDR ligands like the new low calcemic analog ZK156979 have pleiotropic capacities with respect to immune regulation. It is intriguing that these properties are based upon several different molecular mechanisms of cytokine inhibition, while antigen presenting

and T cells can be direct targets of the mother compound calcitriol or the derivative, leading to the inhibition of pathogenic effector T cells. The immunosuppressive activities, described in this in vivo study for ZK156979, coupled with a lack of major side effects once calcemia is under control, might be an auspicious option to be translated into effective immunointervention in IBD as well as in a variety of other models of autoimmune diseases and graft rejection. The advent of calcitriol and its analogues, i.e. ZK156979 presented here, as immunomodulatory agents may open a sound basis to further explore their immunomodulative capacities via the design of novel therapies for autoimmune diseases as IBD.

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Legends for Figures

Figure 1

Structure of the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (A) and the structural 22-ene-25-oxa vitamin D analog ZK156979 (B).

Figure 2

Dose-response profile of the in vivo therapeutic potential of ZK156979 compared to the mother compound calcitriol in Th1-mediated TNBS-colitis. TNBS-treated mice received the calcitriol analogue ZK156979 [0.1 to 2.0 µg/kg] or calcitriol [0.2 µg/kg] i.p. .(A) Body weight (BW)-change on day 3 in % of day 0, (B) clinical activity score (CAS), (C) colon weight (distal 6 cm) (D) colon length was determined on day 3 as described in detail in the Materials and Methods section. Data represent mean ± SEM from three separate experiments (6 mice/group/experiment). * = P<0.05; ** = P<0.01; and *** = P<0.001 vs. TNBS-treated mice.

Figure 3

Macroscopic and microscopic analysis of colons from mice with acute TNBS-colitis. Mice were treated i.p. with calcitriol [0.2 µg/kg] or ZK156979 [0.2 or 2.0 µg/kg], respectively. (A) A representative photograph is shown of colons from day 3 after the instillation of the TNBS-enema. (1) ethanol-treated control, (2) TNBS-treated, (3) TNBS + 0.2 µg/kg calcitriol, (4) TNBS + 0.2 µg/kg ZK156979, (5) TNBS + 2.0 µg/kg ZK156979. (B) Macroscopic score of colitis. Results represent the mean ± SEM from 8 mice per group. (C) Photomicrographs of colon sections after treatment with 45% ethanol (C1); TNBS in 45% ethanol (C2); TNBS + 0.2 µg/kg calcitriol (C3); TNBS + 0.2 µg/kg ZK156979 (C4); TNBS + 2.0 µg/kg ZK156979 (C5). Histopathologic scoring (D); original magnifications 250x. Results are the mean ± SEM from 8 mice per group. * = P<0.05; ** = P<0.01; and *** = P<0.001 vs. TNBS-treated mice.

Figure 4

Effect of calcitriol or ZK156979 on clinical parameters of established ongoing TNBS colitis. The treatment protocol was started on day 3 following the instillation of the haptenating agent. Calcitriol [0.2 µg/kg] or ZK156979 [0.2 µg/kg or 2.0 µg/kg] were administered i.p. starting on day 3 following the TNBS-enema and continued during the next 2 days; then the colon was excised on day 5. The colitis severity was assessed by analysis of BW change on day 3-5 in % of day 0 (A-C); the clinical activity score of colitis is given from day 3-5 (D-F); colon length (G), and colon weight of a 6 cm colon portion (H) has been determined on day 5. Results represent the mean ± SEM from 8 mice per group. * = $P<0.05$; ** = $P<0.01$; and *** = $P<0.001$ vs. TNBS-treated mice.

Figure 5

TNBS-treated mice were medicated i.p. with calcitriol [0.2 µg/kg] or ZK156979 [0.2 or 2.0 µg/kg] from day 0-3 following the instillation of the TNBS-enema. (A) IFN-γ, (B) TNF-α and (C) MPO-activity were determined on day 3. The results represent the mean ± SEM from three separate experiments (8 mice/group/experiment). * = $P<0.05$; ** = $P<0.01$; and *** = $P<0.001$ vs. TNBS-treated mice.

Figure 6

Mice received calcitriol [0.2 µg/kg] or ZK156979 [0.2µg/kg or 2.0 µg/kg] i.p. from day 0-3 following the TNBS-instillation, respectively. (A) A representative immunoblot of the relevant Th1 transcription factor T-bet of either untreated TNBS-colitic mice or colitic TNBS-mice treated with calcitriol [0.2 µg/kg] or ZK156979 [0.2 or 2.0 µg/kg], control animals received 45% ethanol rectally. Two different mouse colon probes per treatment regimen are blotted. (B) Densitometric evaluation of the T-bet immunoblot analysis. The bars are the

mean ± SEM of three separate experiments (n=6) per group. * = P<0.05; ** = P<0.01; and *** = P<0.001 vs. TNBS-treated mice.

Figure 7

Mice were treated i.p. from day 0-3 following the rectal TNBS-instillation with calcitriol [0.2 µg/kg] or ZK156979 [0.2 or 2.0 µg/kg], respectively. (A) IL-4 content of colon protein extracts from day 3 is shown. The results are the mean ± SEM from three different experiments (8 mice per group). (B) Treatment with calcitriol or ZK156979 results in an induction of the anti-inflammatory cytokine IL-10. The bars show the mean ± SEM of three separate experiments (n=6) per group. * = P<0.05; ** = P<0.01; and *** = P<0.001 vs. TNBS-treated mice.

Table 1: Blood calcium and creatinine profile

	Serum calcium [mg/dl]	**Serum creatinine [mg/dl]**
Control	9.72±0.32	0.98±0.09
TNBS [100 mg/kg]	10.08±0.19	0.95±0.06
+ calcitriol [0.2 µg/kg]	11.55±0.35[b]	0.97±0.05[d]
+ ZK156979 [0.2 µg/kg]	9.84±0.07[d]	0.98±0.08[d]
+ ZK156979 [2.0 µg/kg]	9.89±0.25[d]	0.97±0.09[d]

Note. TNBS-treated mice were treated i.p. with calcitriol [0.2 µg/kg] or ZK156979 [0.2 and 2.0 µg/kg] from day 0-3 following the TNBS-enema. Serum calcium and creatinine were determined following a 3 day treatment regimen. Data represent mean ± SEM from 3 separate experiments (8 mice/group/experiment). [b]$p<0.01$; [d]non significant vs. TNBS-treated mice.

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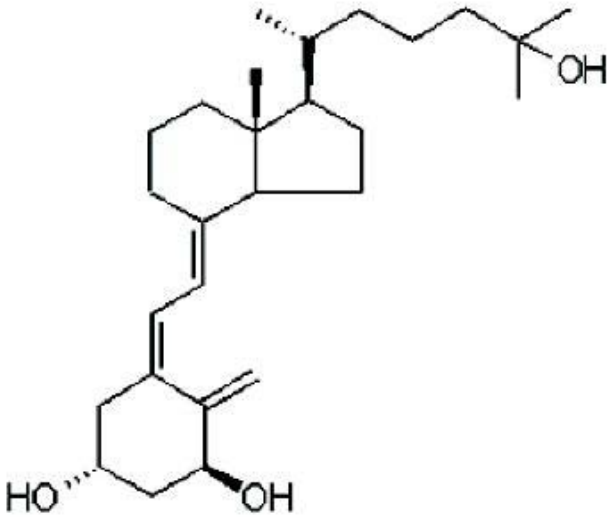
Fig.1

A



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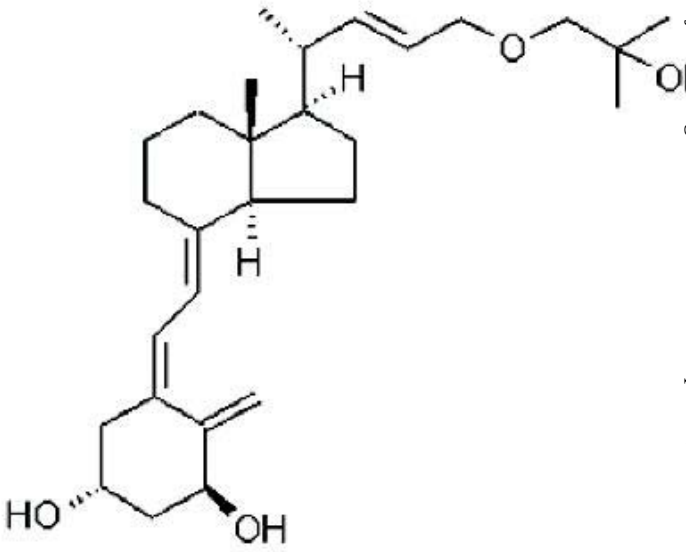

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Fig. 2

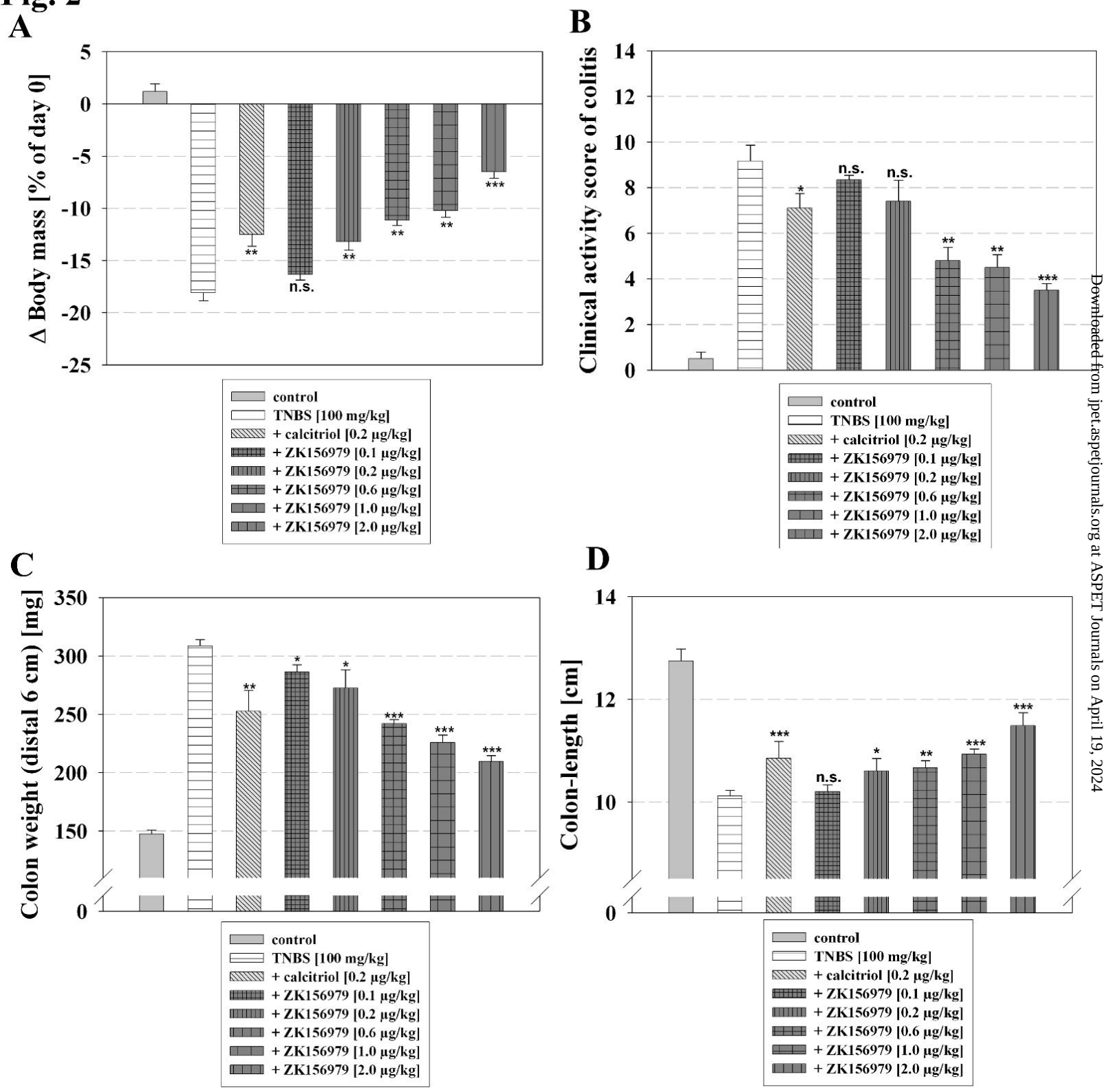

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Fig. 3

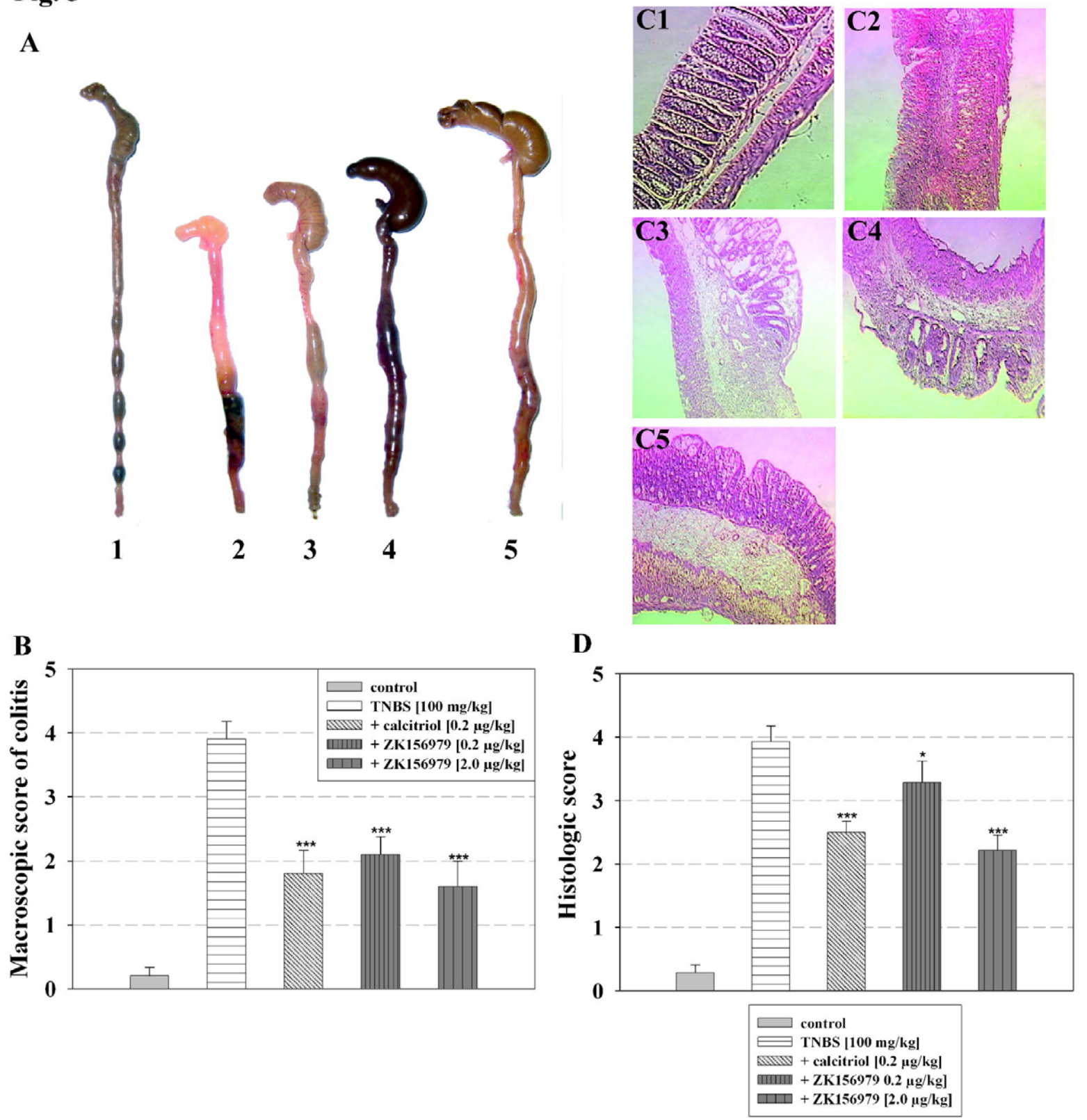

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Fig. 4

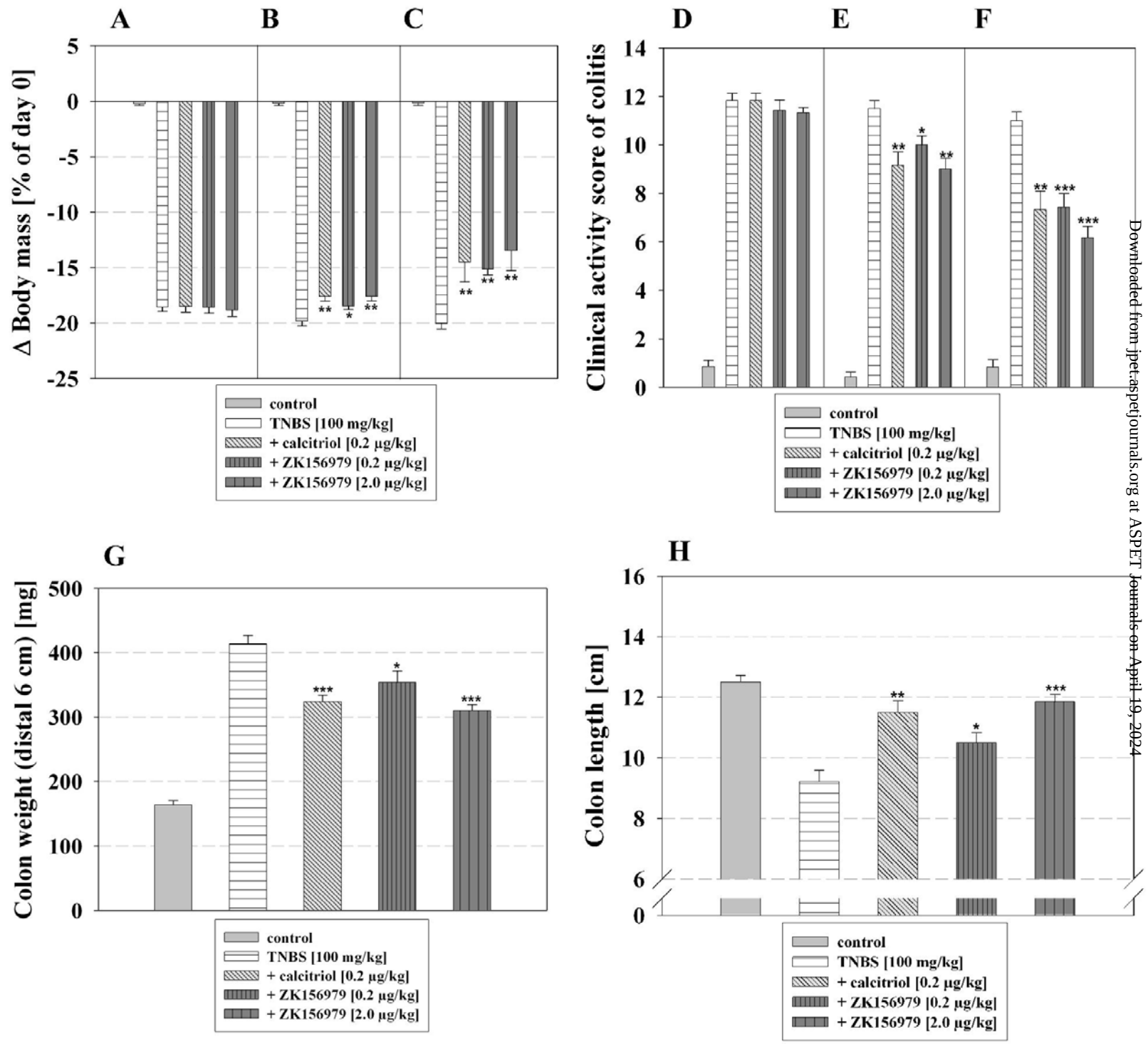

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Fig. 5

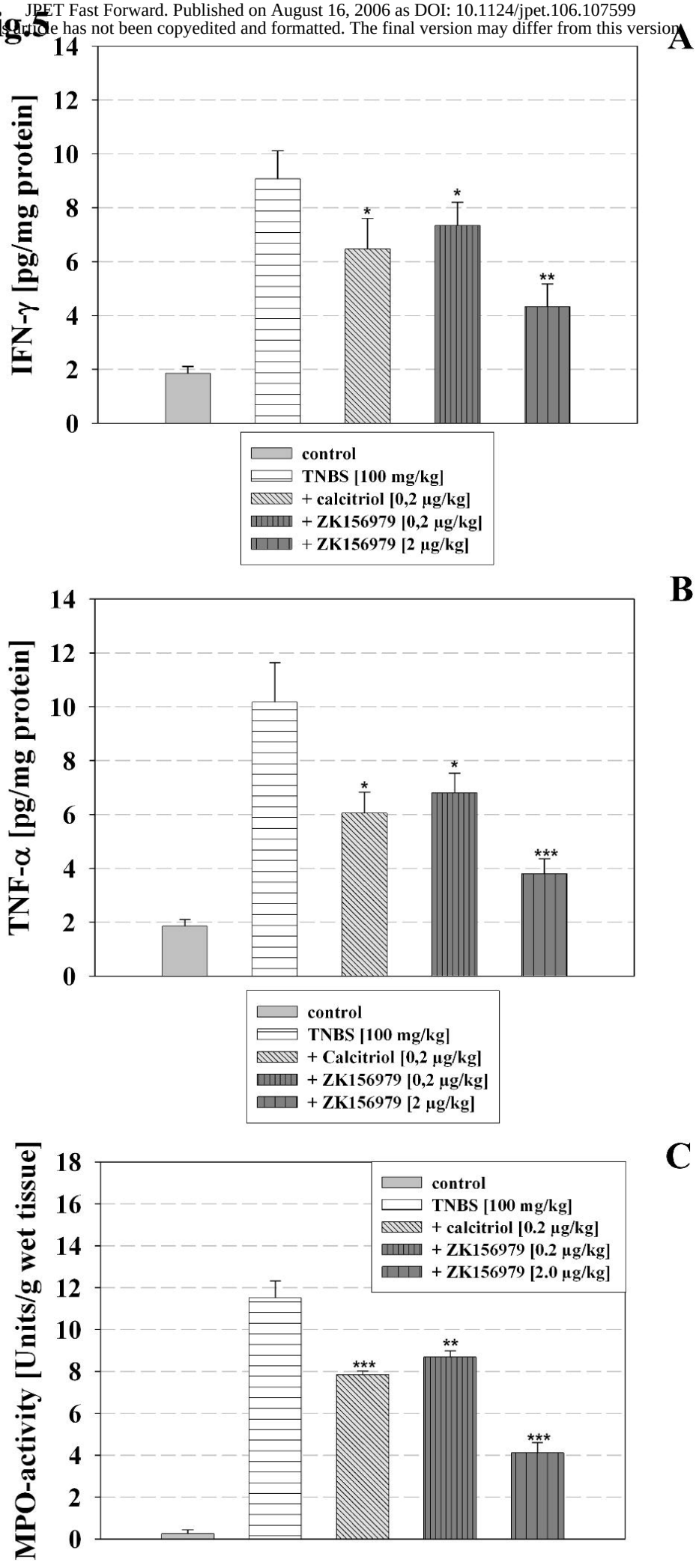

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Fig. 6

TNBS	+	+	+	+	+	+	+	+	-	-
+ calcitriol [0.2 µg/kg]	-	-	+	+	-	-	-	-	-	-
+ ZK156979 [0.2 µg/kg]	-	-	-	-	+	+	-	-	-	-
+ ZK156979 [2.0 µg/kg]	-	-	-	-	-	-	+	+	-	-
ethanol-treated control	-	-	-	-	-	-	-	-	+	+

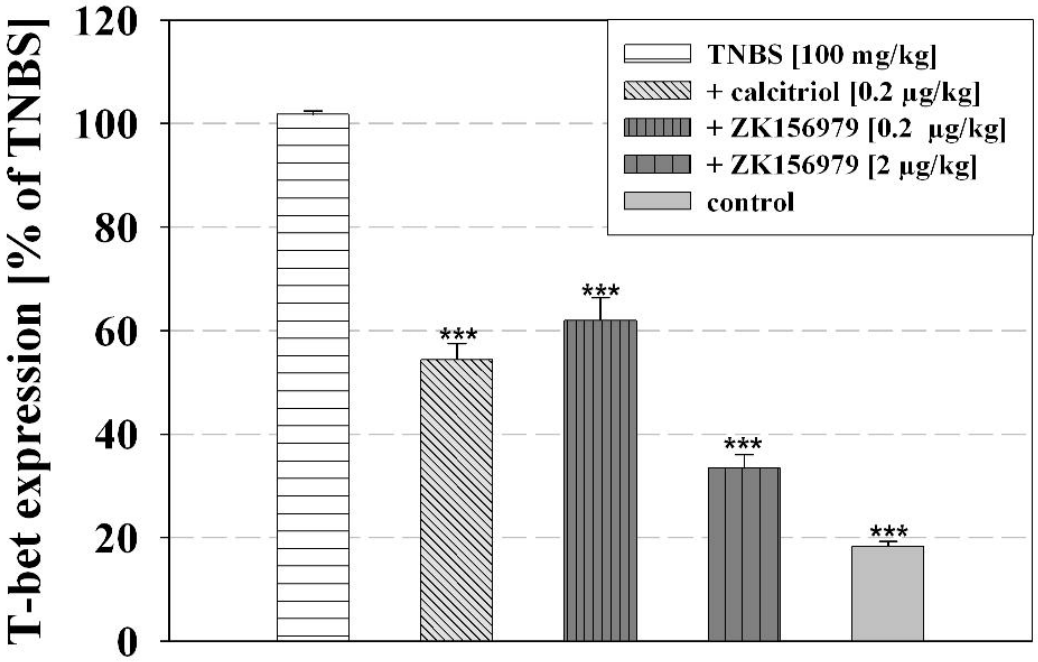

Fig. 7

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