The New Low Calcemic Vitamin D Analog 22-Ene-25-Oxa-Vitamin D Prominently Ameliorates T Helper Cell Type 1-Mediated Colitis in Mice

Carolin Daniel, Heinfried H. Radeke, Nico A. Sartory, Nadine Zahn, Ulrich Zuegel, Andreas Steinmeyer, and Jürgen Stein

First Department of Internal Medicine, Center for Drug Research, Development and Safety (ZAFES) (C.D., N.A.S., N.Z., J.S.) and Pharmazentrum Frankfurt ZAFES (H.H.R.), Johann Wolfgang Goethe University of Frankfurt on Main, Frankfurt, Germany; and Schering Corporate Research, Berlin, Germany (U.Z., A.S.)

Received May 15, 2006; accepted August 10, 2006

ABSTRACT

In addition to its well defined role as a key regulator of calcium and bone metabolism, 1,25-dihydroxyvitamin D₃ (calcitriol) has been established as a potent modulator of immune cell function. Still, because of 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 well preserved immunosuppressive activity in vitro. Here, in vivo colitis was induced by applying a rectal enema of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to male BALB/c mice, and calcitriol (0.2 μg/kg) or ZK156979 (0.1–2.0 μg/kg) was given i.p. from days 0 to 3 or 3 to 5. Body mass and clinical activity score of colitis were recorded daily. Colon tissue was analyzed macroscopically and microscopically, myeloperoxidase activity and cytokine levels [tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-10, and IL-4] were determined by enzyme-linked immunosorbent assay, and T-box transcription factor (T-bet) expression was determined by immunoblot analysis. We found that treatment with ZK156979 clearly reduced the severity of TNBS-induced colitis without exhibiting calcemic effects. Both early and late treatment abrogated body weight loss, diarrhea, and macroscopic intestinal inflammation with a potency comparable with that of calcitriol. The therapeutic effect of ZK156979 was accompanied by a down-regulation of myeloperoxidase activity, TNF-α, IFN-γ, and T-bet expression decreased, whereas local tissue IL-10 and IL-4 protein levels increased. To conclude, our data provide the first clear evidence that ZK156979 exhibits a beneficial prophylactic as well as therapeutic profile in T helper cell type 1-like experimental colitis, offering new therapeutic options for the treatment of human inflammatory bowel diseases.

ABBREVIATIONS: VDR, vitamin D receptor; Th1, T helper cell type 1; Th2, T helper cell type 2; DC, dendritic cell; ZK156979, 22-ene-25-oxa-vitamin D; PBMC, peripheral blood mononuclear cell; CD, Crohn’s disease; TNBS, trinitrobenzene sulfonic acid; MPO, myeloperoxidase; IFN, interferon; TNF, tumor necrosis factor; T-bet, T-box transcription factor; IL, interleukin; IBD, inflammatory bowel disease; GPR30, G protein-coupled receptor 30 involved in rapid nongenomic estrogen-mediated signaling.
ever, the recent finding of a new endosomal high-affinity G-protein-coupled receptor for 17β-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, whereas 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 reduced calcemic activity (Steinmeyer et al., 2000; Mathieu and Adorini, 2002; Zugel et al., 2002; Griffin et al., 2003). The investigation of the 25-oxa series generated a large number of calcitriol analogs exhibiting substantial dissociation between possible immunomodulatory capacities and undesired hypercalcemia. In particular, the combination of the 22-ene situation with the 25-oxa element 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 compound, 22-ene-25-oxa vitamin D (ZK156979) (Fig. 1), may qualify as a promising member of this novel class of vitamin D analogs since 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 pathophysiology may be established as 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 immune response to hitherto unknown agents (Revankar et al., 2005). 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, histopathological, and immune characteristics of CD in humans including the progression to chronic colitis with severe transmural inflammation (Strober et al., 2002).

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

Materials and Methods

Animals

Male, 8-week-old BALB/c mice weighing approximately 20 g were used in all experiments (Charles River Laboratories, Sulzfeld, Germany). All studies were performed with the 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

Thelper type 1-mediated colitis was induced via rectal instillation of the haptenating agent 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 b.wt. to mildly anesthetized 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 after 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 × 10−2 M and kept at −80°C until use. Calcitriol was administered i.p. at a dose of 0.2 µg/kg b.wt. in 0.9% NaCl solution containing 0.085% Myrj53 (Sigma-Aldrich); the solutions were prepared freshly every day. 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 b.wt. in 0.9% NaCl solution containing 0.085% Myrj53, respectively. Two protocols were used. 1) For acute colitis, calcitriol or ZK156979 was administered i.p. 2 h before the instillation of the TNBS enema and during the following 3 days. On day 3, the colon was removed after cervical dislocation under isoflurane anesthesia. 2) For established ongoing colitis, calcitriol or ZK156979 was administered from days 3 to 5 after the instillation of the TNBS enema. On day 5 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
Assessment of Inflammation and Colitis Severity

Clinical Activity Score of Colitis. Body weight 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 who were unaware of the treatment protocol using a scoring system described previously in detail (Hartmann et al., 2000). In brief, the loss of body weight was scored as follows: 0, no weight loss; 1, weight loss of 1 to 5%; 2, weight loss of 5 to 10%; 3, loss of 10 to 20%; and 4, weight loss >20%. Diarrhea (stool consistency) was assessed as follows: 0, normal formed pellets; 2, pasty and semiformal pellets; and 4, liquid stools. Bleeding was assessed as follows: 0, no blood in Hemoccult test; 2, positive Hemoccult results; and 4, gross bleeding from the rectum. The results of these scoring parameters were added to determine a total clinical score ranging from 0 (healthy) to 12 (maximal illness/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.

Macroscore Scoring System. The macroscopic colonic damage was assessed using the scoring system of 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 semiquantitative 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; and 5, ulceration extending over >2 cm. Inflammation was assigned a score of 0 to 5 according to the severity of the transmural inflammation: 0, no inflammation; 1, minimal inflammation extending into the mucosa; 2, moderate inflammation extending into the submucosa and associated erosions, with minimal inflammation of muscularis propria; 3, 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 MPO activity assay according to the method described by 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 (Sigma-Aldrich). MPO (Sigma-Aldrich) 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 remove any fecal matter, snap-frozen in liquid nitrogen, and stored at −80°C until use. The extraction of colonic protein was performed using a cell extraction kit according to the instructions (Active Motif Nuclear extract kit; Rixensart, Belgium). Briefly, the excised colon was washed with ice-cold phosphate-buffered saline and homogenized in ice-cold complete lysis buffer. After an incubation step of 30 min on ice the lysates were centrifuged twice at 4000g and 4°C for 20 min. Aliquots of the resulting extracts were then analyzed for their protein content using the Bio-Rad colorimetric assay according to the method of Bradford (1976) (BioRad Laboratories, Muenchen, Germany) and stored at −80°C until use.

Cytokine Assays

The amounts of murine TNF-α, IFN-γ, IL-4, and IL-10 in the colonic protein lysates were quantified by commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions and adapted to the protein content of the colon tissue probe.

Western Immunoblot Analysis

After the addition of sample buffer to the colonic protein extracts and boiling samples at 95°C for 5 min, 150 µg of total protein lysate was separated with a 10% SDS-polyacrylamide gel (T-bet). Proteins were transferred onto nitrocellulose membranes (Schleicher and 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. The level of proteins was assayed using the appropriate primary antibody (mouse T-bet sc-21749; from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at +4°C. Immunoreactivity was demonstrated by an enhanced chemiluminescence ECL system (GE Healthcare, Buckinghamshire, UK) using an appropriate horseradish peroxidase-conjugated secondary antibody (NA931; from GE Healthcare, Freiburg, Germany). Blots were detected after exposure to Hyperfilm-MP (GE Healthcare). Blots were re-probed with an actin antibody (Santa Cruz Biotechnology). For quantitative analysis, the bands were detected with scanning densitometry, using a CabUVIS scanner and ProViDoc software (Desaga, Wiesloch, Germany).

Statistical Analysis

All data are expressed as means ± S.E.M. Statistical significance of differences between TNBS and calcitriol or ZK156979 treatments 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 3 after the first application of calcitriol or 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 with Calcitriol on Inflammation and Colitis Severity in Acute and Established Ongoing Th1-Mediated TNBS-Induced Colitis

We first investigated the effect of the vitamin D analog ZK156979 on the prevention of the acute TNBS-induced colitis (Th1-model). In this acute model, the first dose of ZK156979 or calcitriol was administered i.p. 2 h before the instillation of the TNBS enema and subsequently during the following 3 days. On day 3, 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 Figs. 2 to 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 with the mother compound calcitriol in this Th1-mediated colitis model, a dose range from 0.1 to 2.0 \( \mu g/kg \) was administered i.p. to male BALB/c mice. Dose-response data for ZK156979 were established after assessment of inflammation, colitis severity, and macroscopic colitis parameters and are shown in Fig. 2, A to D. After TNBS instillation a fast decrease in body weight 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 treated once daily with ZK156979 i.p. on day 3 exhibited a significant reduction of the colitis-associated weight loss correlating with the dose of the analog applied (0.1–2.0 \( \mu g/kg \)) (Fig. 2A).

The clinical activity score of colitis was analyzed as described in detail under Materials and Methods. Colitis was significantly and dose dependently blunted in the ZK156979-treated mice (Fig. 2B). The TNBS enema led to a significant reduction of mean colon length, as assessed on day 3 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 (\( P < 0.001 \) versus the TNBS group, Fig. 2, C and D).

Macroscopic analysis of colons obtained 3 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 (Fig. 3, A and B). To determine macroscopic changes quantitatively, the severity of colonic inflammation and ulceration was graded using criteria for macroscopic scoring as described in detail under Materials and Methods. The administration of the vitamin D analog ZK156979 significantly and

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Calcium (mg/dl)</th>
<th>Serum Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.72 ± 0.32</td>
<td>0.98 ± 0.09</td>
</tr>
<tr>
<td>TNBS (100 mg/kg)</td>
<td>10.08 ± 0.19</td>
<td>0.95 ± 0.06</td>
</tr>
<tr>
<td>+ Calcitriol (0.2 ( \mu g/kg ))</td>
<td>11.55 ± 0.35(^a)</td>
<td>0.97 ± 0.05(^b)</td>
</tr>
<tr>
<td>+ ZK156979 (0.2 ( \mu g/kg ))</td>
<td>9.84 ± 0.07(^a)</td>
<td>0.98 ± 0.08(^b)</td>
</tr>
<tr>
<td>+ ZK156979 (2.0 ( \mu g/kg ))</td>
<td>9.89 ± 0.25(^b)</td>
<td>0.97 ± 0.09(^b)</td>
</tr>
</tbody>
</table>

\(^a\)P < 0.01.\(^b\)Nonsignificant versus TNBS-treated mice.

### Blood calcium and creatinine profile

TNBS mice were treated i.p. with calcitriol (0.2 \( \mu g/kg \)) or ZK156979 (0.2 and 2.0 \( \mu g/kg \)) from days 0 to 3 after the TNBS enema. Serum calcium and creatinine were determined after a 3-day treatment regimen. Data represent means ± S.E.M. from three separate experiments (eight mice per group per experiment).

![Fig. 2. Dose-response profile of the in vivo therapeutic potential of ZK156979 compared with the mother compound calcitriol in Th1-mediated TNBS-induced colitis.](image-url)
dose dependently improved macroscopic scores on day 3 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 (Fig. 3, A and B).

The severity of colonic inflammation and ulceration was further evaluated by histological examinations (Fig. 3C). By day 3 transmural inflammation, characterized by infiltration of inflammatory cells, predominantly neutrophils and lymphocytes, was accompanied by ulcerations, loss of goblet 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 3 days after TNBS instillation significantly (Fig. 3D).

In the next set of experiments we evaluated whether ZK156979, in addition to its ability to prevent acute TNBS-induced colitis, might also be able to reverse established TNBS-induced colitis. To this end, the first dose of ZK156979 or calcitriol was administered on the 3rd day after disease initiation with the TNBS enema and continued subsequently for the next 2 days. The colon was then removed on day 5 after TNBS instillation. With potencies similar to those with calcitriol, ZK156979 treatment also blocked the development of the disease and led to an improvement as indicated by regaining of lost body weight, a reduction in the clinical activity score, and a significant amelioration of the macroscopic signs of colitis (Fig. 4, A–H). These data indicate that ZK156979 is effective not only as an experimental preventive drug but also as a true therapeutic agent for established colitis.

Impact of ZK156979 Compared with Calcitriol on the Inflammatory Response in Acute TNBS-Induced Colitis. Because in vitro we recently demonstrated the substantial anti-inflammatory ability of ZK156979, we investigated

Fig. 4. Effect of calcitriol or ZK156979 on clinical parameters of established ongoing TNBS-induced colitis. The treatment protocol was started on day 3 after the instillation of the haptenating agent. Calcitriol (0.2 μg/kg) or ZK156979 (0.2 or 2.0 μg/kg) was administered i.p. starting on day 3 after the TNBS enema and continued during the next 2 days; the colon was excised on day 5. Colitis severity was assessed by analysis of body weight change on days 3 to 5 in percentage of day 0 (A–C). The clinical activity score of colitis is given from days 3 to 5 (D–F). Colon length (G) and colon weight of a 6-cm colon segment (H) was determined on day 5. Results represent the means ± S.E.M. from eight mice per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus TNBS-treated mice.
in this study, whether ZK156979 could affect the local production of selected inflammatory mediators in mice with acute TNBS-induced colitis in vivo. First, to quantify neutrophil infiltration during ongoing disease we evaluated the effect of ZK156979 on MPO activity in colon extracts 3 days after the instillation of the TNBS enema. In the acute phase of colitis (day 3), colonic MPO activity values were significantly increased compared with those for the ethanol-treated control group (TNBS group 11.5 ± 0.8 versus control group 0.25 ± 0.2; Fig. 5C). In contrast, MPO activities in animals treated with ZK156979 reached levels 25% (0.2 g/kg) and 65% (2.0 g/kg) below those of 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 proinflammatory cytokines in vivo. The administration of ZK156979 led to a significant down-regulation of TNF-α and IFN-γ protein expression (Fig. 5, A and 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 (Fig. 6, A and B).

Next we analyzed the in vivo impact of ZK156979 on the promotion of a Th2 profile, which was indicated by our previous in vitro studies. Both the parent compound calcitriol and ZK156979 markedly up-regulated IL-4 at both doses analyzed while not affecting calcium levels. Moreover, calcitriol and ZK156979 both significantly enhanced the production of the anti-inflammatory cytokine IL-10 compared with that in the untreated TNBS group (Fig. 7, A and 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 phytohemagglutinin-stimulated human PBMCs (Daniel et al., 2005). The substantial amelioration of the clinical parameters of Th1-mediated TNBS-induced 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 after treatment with ZK156979 was further supported by the demonstration 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 (Hypponen et al., 2001; Mahon et al., 2003; Zella and DeLuca, 2003; Cantorna et al., 2004; Peterlik and Cross, 2005). 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). By using animal models, the pleiotropic pathophysiological consequences of insufficient calcitriol supplementation or genetic VDR defects have been emphasized. 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 promise for a wide spectrum of clinical applications of VDR ligands in treatments of inflammatory disorders including rheumatoid arthritis, psoriasis, and 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, 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 potency 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 with the physiological calcitriol-VDR complex (Verlinden et al., 2001; Carlberg, 2003). Further characterization of the molecular events underlying the separation of hypercalcemic and nonhypercalcemic effects of calcitriol agonists may allow 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 100-fold lower hypercalcemic activity compared with the parent compound calcitriol (Steinmeyer et al., 2000).

The central step in calcitriol signaling is the conformational change of the ligand binding domain of VDR and the resulting exchange of protein-protein interaction partners. DNA-bound VDR-retinoid X receptor 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 nonagonistic conformation of the ligand-binding domain of VDR 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 deriv-
tives function as agonists, which also holds true for ZK156979 used here.

Inhibition of factors leading to Th1 polarization after 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 proinflammatory 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 naive 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, DCs, and a subtype of regulatory T cells (Boonstra et al., 2001; Barrat et al., 2002; Griffin et al., 2003). Although 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; Pichler et al., 2002; Staeva-Vieira and Freedman, 2002).

In a previous in vitro study using human PBMCs, we clearly demonstrated prominent immunosuppressive capabilities for ZK156979, resulting in strong inhibition of the phytohemagglutinin-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 with that of calcitriol (Daniel et al., 2005). In addition, in these in vitro experiments ZK156979, in concordance with calcitriol, also affected the Th2 response, leading to significant increases of IL-4 and IL-10. The latter observations are consistent with the findings in this study demonstrating that the immunosuppressive capabilities of ZK156979 are transferable to the in vivo model of Th1-mediated TNBS-induced 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 markers for neutrophil infiltration and proinflammatory cytokines. However, in contrast with 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 counterbalanced by either Th2 or regulatory cytokines, possess potent proinflammatory 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 and IFN-γ production and T-bet expression, whereas 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 abilities with respect to immune regulation. It is intriguing that these properties are based upon several different molecular mechanisms of cytokine inhibition, whereas 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 analogs, i.e., ZK156979 presented here, for use as immunomodulatory agents may provide a sound basis to further explore their immunomodulative capacities via the design of novel thera-

References


Canterna MT, Zhu Y, Fricou M, and Wittke A (2004) Vitamin D status, 1,25-


Manolagas SC, Provendini DM, and Tsooukas CD (1965) Interactions of 1,25-


Address correspondence to: Dr. Jürgen Stein, 1st Department of Internal Medicine, Division of Gastroenterology and Clinical Nutrition, Johann Wolfgang Goethe University, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. E-mail: j.stein@em.uni-frankfurt.de