Selective Glucocorticoid Receptor Agonists for the Treatment of Inflammatory Bowel Disease: Studies in Mice with Acute Trinitrobenzene Sulfonic Acid Colitis

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ABSTRACT

Despite being a mainstay of inflammatory bowel disease (IBD) therapy, glucocorticoids (GCs) still carry significant risks with respect to unwanted side effects. Alternative drugs with a more favorable risk/benefit ratio than common GCs are thus highly desirable for the management of IBD. New and supposedly selective glucocorticoid receptor (GR) agonists (SEGRAs), with dissociated properties, have been described as promising candidates for circumventing therapeutic problems while still displaying full beneficial anti-inflammatory potency. Here, we report on compound A [CpdA; (2-((4-acetophenyl)-2-chloro-N-methyl)ethylammonium-chloride)] and N-(4-methyl-1-oxo-1H-2,3-benzoxazine-6-yl)-4-(2,3-dihydrobenzofuran-7-yl)-2-hydroxy-2-(trifluoromethyl)-4-methylpentanamide (ZK216348), two GR agonists for the treatment of experimental colitis. Their therapeutic and anti-inflammatory effects were tested in the acute trinitrobenzene sulfonic acid-mediated colitis model in mice against dexamethasone (Dex). In addition to their influence on immunological pathways, a set of possible side effects, including impact on glucose homeostasis, steroid resistance, and induction of apoptosis, was surveyed. Our results showed that, comparable with Dex, treatment with CpdA and ZK216348 reduced the severity of wasting disease, macroscopic and microscopic damage, and colonic inflammation. However, both SEGRAs exhibited no GC-associated diabetogenic effects, hypothalamic pituitary adrenal axis suppression, or development of glucocorticoid resistance. In addition, CpdA and ZK216348 showed fewer transactivating properties and successfully dampened T helper 1 immune response. Unlike ZK216348, the therapeutic benefit of CpdA was lost at higher doses because of toxic apoptotic effects. In conclusion, both SEGRAs acted as potent anti-inflammatory agents with a significantly improved profile compared with classic GCs. Although CpdA revealed a narrow therapeutic window, both GR agonists might be seen as a starting point for a future IBD treatment option.

Introduction

Glucocorticoids (GCs) are widely used as potent immunosuppressive and anti-inflammatory agents and represent an important cornerstone for the treatment of inflammatory bowel disease (IBD) (Martins and Peppercorn, 2004; Rogler, 2010). Their therapeutic use, however, is restricted by the occurrence of GC-associated severe adverse systemic effects, including osteoporosis, muscle wasting, insulin resistance, lipid redistribution, and the inhibition of wound repair, which increase with dose and duration of treatment (Stanbury and Graham, 1998; Rogler, 2010). Moreover, the frequently observed development of steroid resistance and de-
endence completes the complex of problems for patients subjected to GC therapy (Faubion et al., 2001).

Both the anti-inflammatory and the metabolic effects of GCs depend on the GR receptor (GR). Upon ligand binding, the GR undergoes a structural alteration, which, in turn, after translocation into the nucleus, enables initiation (transactivation) or suppression (transrepression) of target gene transcription. To exert its transactivation function, the GR binds as a homodimer to glucocorticoid response elements (GRREs) in the promoter of GC-responsive genes, whereas for transrepression GR monomers negatively interfere with the activity of transcription factors, e.g., NFκB, activator protein-1 (AP-1), or cAMP response element-binding protein (CREB) (Heck et al., 1994; Barnes, 2006). Although repression of gene regulation by blocking the functionality of proinflammatory transcription factors largely accounts for the anti-inflammatory actions of GCs, activation of target genes involved in the metabolism of glucose, protein, fat, muscle, and bone is implicated in adverse effects (Jacobson et al., 2005; Barnes, 2006; Macfarlane et al., 2008).

The search for safer GCs with an improved therapeutic index experienced a turn when it was discovered that the mechanisms of transactivation and transrepression are functionally separate events, and the absence of DNA-binding ability of the GR did not result in the loss of the GR’s anti-inflammatory action (Reichardt et al., 1998a). Exploitation of this discovery would allow the preservation of beneficial effects while reducing dose-related side effects by finding GR ligands, selectively binding to and modifying GR conformation toward gene suppression, thus altering the profile of gene expression. Indeed, efforts to identify improved or innovative GR ligands have resulted in the introduction of selective GR agonists (SEGRAs) (Miner et al., 2005; Schäcke et al., 2007), and several candidates have been reported. In 2004 (Schäcke et al., 2004), N-(4-methyl-1-oxo-1H-2,3-benzoxazine-6-yl)-4-(2,3-dihydrobenzofuran-7-yl)-2-hydroxy-2-(trifluoromethyl)-4-methylpentanamide (ZK216348) was identified as a nonsteroidal ligand with affinity for both the GR and the progesterone receptor (PR) and, although in an antagonistic mode, reported binding to the mineralocorticoid receptor (MR). ZK216348 displays SEGRA properties because it possesses anti-inflammatory activity comparable with prednisolone for both systemic and topical application in a murine model of skin inflammation yet it displays a significantly lower risk for the induction of diabetes in vivo (Schäcke et al., 2004) and less potential to cause bone loss than standard GCs in vitro (Humphrey et al., 2006).

Among the SEGRAs described as GR ligands, compound A [CpdA; (2-((4-acetophenyl)-2-chloro-N-methyl)ethylamino)phenyl]-17β-hydroxy-17α-propynylestra-4,9-dien-3-one) has been characterized as a steroid receptor ligand with high affinity for GR (De Bosscher et al., 2005) and efficient dissociation between the mechanism of GRs transcriptional activation and repression in vitro and in vivo, while not showing typical GC-associated side effects (De Bosscher et al., 2005; Dewint et al., 2008; Yemelyanov et al., 2008; Gossye et al., 2010; Wüst et al., 2009; Zhang et al., 2009; van Loo et al., 2010).

In summary, although controversial, but on the basis of additional publications that report the successful application of different GR modulators (Owen et al., 2007; Schäcke et al., 2007; López et al., 2008), the dissociation concept of SEGRAs still has promising potential for the treatment of inflammatory conditions such as IBD. Therefore, in a TNBS model of acute colitis we investigated the GR agonists CpdA and ZK216348, which are classified as SEGRAs. By clearly segregating unwanted side effects from beneficial effects our study might support the optimization of these therapeutics toward a future treatment option for IBD.

**Materials and Methods**

**Induction of Experimental Colitis.** Male, 6- to 8-week-old BALB/c mice weighing on average 20 g were obtained from Harlan Laboratories (Eysstrup, Germany) and housed under normal laboratory conditions with circadian light/dark cycles and free access to standard mouse chow and tap water. The Regional Council’s Ethics Committee for Animal Experimentation (Darmstadt/Hessen, Germany; F134/04) approved all experiments. After an initial adaptation period of 4 days, colitis was induced by intraluminal injection of TNBS (2,4,6-TNBS; Sigma-Aldrich, Hamburg, Germany) at a concentration of 100 mg/kg body weight (BW). A solution of 2% TNBS in 45% ethanol was delivered to slightly anesthetized mice through a flexible catheter 4 cm proximal to the anal verge. To prevent leakage of the TNBS solution, mice were held in a vertical position for 1 min after the installation of the TNBS enema. Control animals received 45% ethanol alone by using the same technique.

**Study Design and Therapy Protocol.** Dexamethasone (Sigma-Aldrich) was dissolved in PBS and administered intraperitonealy at a dose of 1 mg/kg BW. Compound A (Alexxis Biochemicals, Grünberg, Germany) (De Bosscher et al., 2005) was dissolved freshly in 45% ethanol alone by using the same technique.

**ABBREVIATIONS:** GC, glucocorticoid; GR, GC receptor; SEGRA, selective GR agonist; BW, body weight; COX, cyclooxygenase; CpdA, compound A; Dex, dexamethasone; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FL, full length; G-6-P, glucose-6-phosphatase; GRE, glucocorticoid response element; HEK, human embryonic kidney; HPA, hypothalamic pituitary adrenal; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; IBD, inflammatory bowel disease; IBD, inflammatory bowel disease; LPS, lipopolysaccharide; Luc, luciferase; MCP-1, monocyte chemotactic protein-1; MLN, mesenteric lymph node; MPO, myeloperoxidase; MR, mineralocorticoid receptor; NFκB, nuclear factor-κB; PARP, poly(ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PEPCK, phosphoenolpyruvate carboxykinase; PCR, polymerase chain reaction; qPCR, quantitative PCR; PR, progesterone receptor; TAT, tyrosine aminotransferase; Th, T helper; TNBS, trinitrobenzene sulfonic acid; TFN, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; ZK216348, N-(4-methyl-1-oxo-1H-2,3-benzoxazine-6-yl)-4-(2,3-dihydrobenzofuran-7-yl)-2-hydroxy-2-(trifluoromethyl)-4-methylpentanamide; RU-486, 11-β-p-(di-methylamino)phenyl)-17β-hydroxy-17α-propynylestra-4,9-dien-3-one.
ZK216348 (Schäcke et al., 2004) (a kind gift of Dr. H. Schäcke, Bayer Pharma AG, Berlin, Germany), dissolved 3/5/92 in ethanol/dimethyl sulfoxide/peanut oil, was administered subcutaneously at doses of 1 or 10 mg/kg BW. For the protocol of acute ongoing colitis, Dex, CpdA, and ZK216348 were applied 2 h before the instillation of the TNBS enema (midday) and thereafter every 24 h for another 2 days. Mice were sacrificed on day 3 post-TNBS injection, 24 h after the last Dex or SEGRA treatment.

**Clinical Activity Score.** For the assessment of the clinical severity of colitis, mice were given a clinical disease score ranging from 0 (healthy) to 12 (maximal activity of colitis) based on the following characteristic parameters: body weight loss, stool consistency, and the presence of occult/overt blood in the stool. Each parameter was determined daily by using a scoring system described previously (Daniel et al., 2006).

**Myeloperoxidase Activity Assay.** Colon myeloperoxidase (MPO) activity was evaluated to monitor the degree of myeloid cell infiltration into the gastrointestinal tissue and was assayed as described previously (Bradley et al., 1982). MPO (Sigma-Aldrich) was used to prepare a standard curve. Values are expressed as MPO units/gram of wet tissue.

**Macroscopic Analysis and Extent of Inflammation in the Colon.** Postmortem the entire colon (cecum to anus) was removed and rinsed with ice-cold PBS. Measurements of the full length and weight of the distal 6 cm were used as indirect markers of disease-associated intestinal wall thickening, correlating with the intensity of inflammation, and to calculate the colonic length/weight ratio. Subsequently, assessment of the macroscopic changes was performed by observers blinded to the treatment, using a semiquantitative scoring system (Daniel et al., 2006).

**Histological Analysis of the Colon.** Rings of the descending part of the colon, obtained 3 cm above the anus, were fixed in 4% neutral buffered formalin phosphate, embedded in paraaffin, sectioned (3 μm), and stained with hematoxylin and eosin according to standard techniques. For histological scoring tissues were examined by pathologists unaware of the treatment, using a protocol described previously (Dielemann et al., 1998) with the following modification: amount of inflammation (based on polymorphonuclear neutrophil infiltration) ranging from 0 to 4 (0, none; 1, slight; 2, minimal; 3, moderate; 4, severe). All values were added to a sum with a maximum possible score of 44.

**Gene Expression Analysis Using Real-Time PCR.** Total RNA of mouse colon and liver tissues was isolated by the TRIzol reagent as described by the manufacturer (Invitrogen, Darmstadt, Germany). Two micrograms of total RNA underwent DNase I digestion (Fermentas, St. Leon Rot, Germany) and were subsequently reverse-transcribed by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany). Mouse-specific primers for IL-1α, TNF-α, COX-2, tyrosine aminotransferase (TAT), glucose-6-phosphatase (G-6-P), and phosphoenolpyruvate carboxykinase (PEPKC) were designed by using the Primer Express Program (Applied Biosystems, Darmstadt, Germany). Real-time PCR for gene expression was performed from cDNA using the Power SYBR Green PCR Master Mix with AB StepOnePlus (Applied Biosystems) or iCycler (Bio-Rad Laboratories) sequence detector systems under the standard protocol in a volume of 25 μl. Acidic ribosomal phosphoprotein P0 (34B4) was used as an endogenous housekeeping gene to normalize the results. The relative mRNA expression of each studied gene was calculated with the comparative ΔCt method using the formula 2−ΔΔCt.

**Western Blot Analysis.** Protein extracts from colon samples (obtained 2 and 4 cm above the anus and pooled), PBMCs, MM6 cells, and activated mesenteric lymph nodes (MLNs) were prepared by using a cell extraction kit (Active Motif Nuclear Extract Kit; Active Motif, Rixensart, Belgium) according to the instructions of the manufacturer and analyzed for protein content with bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Bonn, Germany). For SDS-polyacrylamide gel electrophoresis and Western blotting refer to the protocol described previously (Daniel et al., 2006). Visualization and quantification of protein bands were carried out with the Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

**TAT Activity.** TAT activity in liver tissue was evaluated 6 h after compound administration to mice. The animals were killed, and a 10-mm-diameter liver biopsy was used for the determination of TAT according to the spectrophotometric method of Diamondstone, as described previously (Schäcke et al., 2004). TAT activity was normalized for hepatic protein content, and TAT induction was defined as fold change of TAT activity over vehicle-treated animals.

**Blood Glucose, Adrenocorticotropic Hormone, and Hepatic Glycogen Determination.** After an overnight fasting period, mice were treated either with Dex, CpdA, or ZK216348 for 6 h. Blood samples were taken by puncture of the vena facialis, and blood glucose levels were determined by using a blood glucose meter (Bayer Healthcare AG, Berlin, Germany). After centrifugation in heparinized tubes, plasma levels of adrenocorticotropic hormone were determined by ELISA, specific for mouse adrenocorticotropic hormone (Hoelzel Diagnostika, Cologne, Germany), following the manufacturer’s manual. Liver biopsies were harvested for evaluation of hepatic glycogen following the previously described protocol (Kepler and Decker, 1994).

**Isolation and Culture of Mesenteric Lymph Node CD4+ T Cells.** After sacrifice, MLNs closest to the inflamed intestine were removed. Single-cell suspensions were prepared by pressing MLNs through a 40-μm nylon cell strainer (BD Biosciences, Heidelberg, Germany) using the plunger of a 1-ml syringe. After washing the mash three times with RPMI 1640 medium containing 10% FCS and a centrifugation step for 10 min at 500g at room temperature, MLN CD4+ T cells were isolated by positive selection using the anti-CD4 (L3T4; Miltenyi Biotec, Bergisch-Gladbach, Germany) magnetic cell sorting system according to the manufacturer’s instructions. The CD4+ T cells (>96% by FACS) were suspended in RPMI 1640 media (supplemented with 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.05 mM 2-mercaptoethanol, and 10% FCS; Invitrogen) and cultured at a concentration of 0.25 × 10⁶ cells/ml in 96-well plates (200 μl/well) precoated with anti-CD3 monoclonal antibody in the presence of 1 μg/ml anti-CD28 monoclonal antibody (both BD Biosciences). After incubation for 48 h at 37°C in 5% CO₂, the supernatants were collected and stored at −70°C until analysis using the ELISA kits for mouse IL-2, IL-4, and IFN-γ (Ready-SET-Go!; eBiosciences, Frankfurt, Germany).

**Isolation, Culture, and Stimulation of PBMCs and MM6.** Human PBMCs were isolated by Ficoll-Hypaque (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) density gradient centrifugation from the blood of healthy donors at the Blutspendedienst Frankfurt/Main, Frankfurt, Germany. All donors agreed by written informed consent. Before stimulation with LPS (1 μg/ml; Sigma-Aldrich), 2 × 10⁶ PBMC or MM6 cells were cultured in RPMI 1640 media (10% FCS, 100 U/ml penicillin/streptomycin, 5 mM HEPES, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol; Invitrogen) for 16 h after pretreatment with Dex, CpdA, or ZK216348 as indicated. Medium with substances was changed every 24 h. Cytokine levels of IL-8, IL-6, and MCP-1 in supernatants were analyzed by using the ELISA kits for mouse IL-2, IL-4, and IFN-γ (Ready-SET-Go!; eBiosciences, Frankfurt, Germany).

**Detection of Apoptosis.** Paraaffin-embedded cross-sections of the distal colon (4 μm; obtained 3 cm above the anus) from 3 days post-TNBS mice underwent TUNEL staining for the detection of apoptotic cells (Apoptag; Millipore, Schwalbach, Germany). Deparaffined slides were treated with protease K (20 μg/ml) for 20 min, after which free 3’ DNA ends were labeled by using terminal deoxynucleotidyl transferase with digoxigenin-dNTPs at 37°C for 1 h. After three washes in PBS, tissue slides were stained for 30 min with rhodamine-labeled antidigoxigenin at room temperature. Counterstaining was performed after four washes in PBS with Hoechst 33258 (1 μg/ml; Sigma-Aldrich). The visualization of red fluorescence TUNEL-positive cells, overlaid with blue fluorescence cell nuclei, was performed at 40× magnification with an Olympus IX71 microscope (Olympus, Hamburg, Germany). To
In vivo activities of CpdA and ZK216348 were characterized by using the TNBS-induced Th1-mediated model of colitis. To assess whether they could exert anti-inflammatory and immune-modulatory activity and thus prevent the signs of acute TNBS-induced colitis, mice administered with TNBS were treated over 3 days either with CpdA, ZK216348, or Dex representing a classic GC. Mice with TNBS-induced colitis developed severe diarrhea accompanied by an extensive wasting disease, characterized by BW loss, enhanced clinical activity score, increased MPO activity, and increased colonic weight/length ratio, compared with control groups, which received only an intrarectal injection of 45% ethanol and showed no clinical disease symptoms over the observation period. Treatment of TNBS colitis with Dex, CpdA, and ZK216348 clearly resulted in an attenuation of local and systemic signs of colitis as assessed by animal weight loss, colonic MPO activity, and improvement in the severity of diarrhea and wasting disease, combined in the clinical activity score of colitis and colonic weight/length ratio. In contrast to ZK216348, which acted dose-dependently, treatment of colitis proved to be more effective with administration of the lower dose of CpdA (1 mg/kg) (Fig. 1). In fact, injection of CpdA at 10 mg/kg BW severely exacerbated the physiological condition of the animals. In comparison to mice receiving Dex, ZK216348, or CpdA at a lower dose, CpdA at 10 mg/kg BW resulted in reduced locomotion, crouching, bristly skin, and apparent abdominal pain.

CpdA and ZK216348 Impede Transmural Inflammatory Cell Infiltration and Colonic Damage. Macroscopic analysis of colons 3 days after TNBS administration indicated remarkable hyperemia, necrosis, and inflammation in contrast to ethanol-treated mice, in which almost no macroscopic damage was observed. Severity of the macroscopic damage in mice treated with CpdA or ZK216348 was significantly lower than that of TNBS-treated animals, with the best results seen at doses of 1 mg/kg CpdA and 10 mg/kg ZK216348 (Fig. 2, A and B). Further histological examination of colon sections from TNBS-treated mice revealed severe mucosal infiltration by inflammatory cells and tissue destruction, whereas administration of CpdA or ZK216348 resulted in a lower extent of inflammation and decreased the histological score in treatment doses similarly observed for the parameters described above (Fig. 2, C and D).

CpdA and ZK216348 Attenuate Colonic Inflammatory Response. Furthermore, administration of CpdA (1 mg/kg) and ZK216348 dose-dependently attenuated the TNBS-induced increase of colonic expression of inflammatory mediators, including TNF-α, IL-1β, and COX-2, which was comparable with Dex treatment (Fig. 3A). Because many of these inflammatory mediators are regulated by NF-κB, a proinflammatory transcription factor well known to be up-regulated in the inflamed mucosa of patients with IBD (Schreiber et al., 1998), and GCs are known to strongly inhibit NF-κB activation, we checked for its presence in colonic tissue. It was found that NF-κB/p65 protein expression was enhanced in mice with TNBS colitis, whereas under treatment with Dex, CpdA, or ZK216348, increases in NF-κB/p65 levels were greatly dampened (Fig. 3B).

CpdA and ZK216348 Are GR Modifiers with Lower Transactivation Potential. Monitoring TAT mRNA expression and activity provides a good tool for investigating dissociation properties of SEGRAs in vivo, because TAT gene transcription during GC treatment is initiated preemi-
nantly by the GRE-driven transactivation mechanism. In our experiments a single dose of Dex increased TAT mRNA expression up to 5-fold over vehicle controls. This contrasted with the administration of CpdA and ZK216348, which in comparison to Dex showed less induction of the TAT gene (Fig. 4A). Furthermore, the Dex-induced increase in hepatic TAT mRNA expression also resulted in accelerated TAT enzyme activity, which was not affected by treatment of mice with SEGRAs (Fig. 4B).

Taking into account that CpdA, in addition to GR, has been shown to influence AR responses (Yemelyanov et al., 2008) and ZK216348 shows further binding affinity for the PR and MR (Schäcke et al., 2004), the contribution of CpdA and ZK216348 to the transcriptional activity of the GR was investigated more generally. Using reporter gene assay and a GRE-driven promoter construct (pGRE-Luc) transiently transfected to HEK293T cells in comparison with CpdA or ZK216348 a significant higher increase in luciferase activity could be observed in the presence of Dex, which was abolished by coincubation of the GR antagonist RU-486. In addition, when given before Dex, CpdA and ZK216348 were able to functionally compete for GR binding, leading to lower transcription levels of the pGRE-Luc construct. This suggests the involvement of a transcriptional GR response in the presence of CpdA and ZK216348 at least in vitro (Fig. 4C). Participation of an active GR in the anti-inflammatory response of CpdA and ZK216348 was further investigated in Caco-2 cells, where the TNF-α-induced expression of the proinflammatory cytokine IL-8 was suppressed in the presence of Dex, CpdA, or ZK216348 and could be reversed by the addition of RU-486 (Fig. 4D). These data suggest that CpdA and ZK216348 are interacting with the GR as agonists, which then might allow dissociating transactivation from GR-mediated transrepression. Although RU-486 exhibits activity as a GR and PR antagonist and CpdA has unique chemical properties (i.e., rapid aziridine formation at physiological pH) (Swart et al., 2003; Wüst et al., 2009), it is not possible to securely state that the observed dissociating effects of CpdA and ZK216348, especially in complex in vivo systems, are exclusively GR-dependent but rather involve the GR in some manner, leaving us to attempt more precise exploration of the complex pharmacology of SEGRAs, which is necessary for their translation into clinical benefit.

CpdA and ZK216348 Attenuate the Activation of the Th1 Profile of Mesenteric Lymph Node CD4+ Cells. Although most of the disease models used for in vivo SEGRA research involve T cell-mediated inflammatory response, only a few data exist regarding the effects of SEGRAs on T-cell function or differentiation and their contribution to the adaptive immune response. In BALB/c mice, TNBS colitis is characterized by a Th-1-driven immune response. To analyze

Fig. 1. Effects of Dex or SEGRA treatment on clinical parameters of acute TNBS colitis. Mice were induced for colitis with TNBS and treated intraperitoneally or subcutaneously with Dex or SEGRAs. Body weight change (A), clinical activity score (B), MPO activity (C), and colonic weight/length ratio (D) were evaluated on day 3 after TNBS induction of colitis. Results represent mean ± S.E.M. of 10 mice per group. *, p < 0.005; **, p < 0.01; ***, p < 0.001 versus TNBS-treated mice.

A

B

C

D

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the phenotype of effector T cells in TNBS colitis under Dex, CpdA, or ZK216348 treatment, CD4⁺ T lymphocytes were isolated from MLNs and ex vivo stimulated. In T cells of mice with TNBS colitis, production of the proinflammatory Th1 cytokines IL-2 and IFN-γ was augmented, correlating with the severity of clinical parameters of colitis. CpdA and ZK216348 treatment caused a down-regulation of both cytokines (Fig. 5, A and B). To address the question of whether the therapeutic effect of CpdA or ZK216348 is clinically restricted to Th1-mediated disease, IL-4 production and the expression of the relevant Th2 transcription factor GATA3 were assessed. The release of the Th2 cytokine IL-4 by SEGRA treatment of TNBS colitis remained largely unchanged (Fig. 5C). However, an increase in GATA3 protein expression after Dex, 1 mg/kg CpdA, and ZK216348 treatment in comparison with TNBS treatment alone could be observed (Fig. 5D). Together with the inhibitory impact CpdA and ZK216348 exert on Th1 effector functions, this simultaneous moderate induction of a Th2 immune response may further explain the anti-inflammatory effect of both substances.

**CpdA and ZK216348 Do Not Induce Hyperglycemia or Suppress the Hypothalamic Pituitary Adrenal Axis.**

GCs are known to increase blood glucose concentrations through transactivation-mediated gene expression of gluconeogenic enzymes (Goldstein et al., 2002). In overnight-
fasted BALB/c mice, 6 h after injection of Dex, blood glucose levels were significantly higher compared with vehicle-treated animals (Fig. 6A). The Dex-induced increase in circulating glucose levels correlated with an increase in mRNA expression of the GC-driven key gluconeogenic enzymes G-6-P or PEPCK (Fig. 6, B and C). In line with previous reports, CpdA or ZK216348 administration at the tested concentrations led neither to a significant increase in blood glucose nor to mRNA expression of liver enzymes. A second classic hepatic response to GCs is glycogen deposition. Dex treatment led to a rapid accumulation of glycogen levels, a storage device for newly synthesized glucose. Animals receiving CpdA or ZK216348 showed no elevated hepatic glycogen formation (Fig. 6D). These acute in vivo studies demonstrate that both substances do not mimic GC-induced metabolic responses involved in glucose homeostasis. A second classic hepatic response to GCs is glycogen deposition. Dex treatment led to a rapid accumulation of glycogen levels, a storage device for newly synthesized glucose. Animals receiving CpdA or ZK216348 showed no elevated hepatic glycogen formation (Fig. 6D). These acute in vivo studies demonstrate that both substances do not mimic GC-induced metabolic responses involved in glucose homeostasis. A second classic hepatic response to GCs is glycogen deposition. Dex treatment led to a rapid accumulation of glycogen levels, a storage device for newly synthesized glucose. Animals receiving CpdA or ZK216348 showed no elevated hepatic glycogen formation (Fig. 6D). These acute in vivo studies demonstrate that both substances do not mimic GC-induced metabolic responses involved in glucose homeostasis.

**CpdA and ZK216348 Do Not Induce GC Resistance In Vitro.** Treatment with GCs over longer periods of time often results in the development of GC resistance, where patients fail to respond even to high doses of GCs. It has been proposed that the down-regulation of GR levels observed after ligand treatment is responsible for this effect. We therefore studied the effect of Dex and SEGRA treatment on the down-regulation of the GR expression in MM6 cells and PBMCs. Exposure of cells to Dex led to a drastic reduction in GR protein levels over time, whereas this effect could not be observed in cells incubated with CpdA or ZK216348, in MM6 or PBMCs (Fig. 7, A and B). Furthermore, as already investigated in fibroblast-like synoviocytes (Gossye et al., 2010), the Dex-induced decrease in GR protein levels is accompanied by a simultaneous reduction of the anti-inflammatory capacity of GCs. This could be confirmed in our experimental setup, where not only Dex, but also CpdA and ZK216348, were able to reduce the LPS stimulated increase in the pro-inflammatory cytokines IL-6, IL-8, and MCP-1. However, in accordance with its effect on GR expression status, the repression function of Dex was substantially lost in cells incu-
bated for longer time periods. It is noteworthy that both GR agonists showed a greater anti-inflammatory potency compared with the classic GC Dex (Fig. 7C).

CpdA and ZK216348 Effects on Induction of Apoptosis. Alerted by the findings of Wüst et al. (2009) concerning the toxicity of CpdA in higher doses, and supported by our observations, we were curious as to whether the pathogenic outcome of CpdA (10 mg/kg) treatment in the acute TNBS-colitis model might be caused by a proapoptotic effect of CpdA. Our in vitro investigations for the apoptotic potential of CpdA and ZK216348 revealed, after incubation of human PBMCs with CpdA in concentrations ≥20 μM, a substantial induction of apoptosis on the overall cell population including monocytes and lymphocytes (Fig. 8A and data not shown), which was more prominent than the observed apoptotic effect of Dex. ZK216348 showed no effect in all tested concentrations (Fig. 8A). Applying the TUNEL assay on sections of mice with TNBS colitis and Western blot technique, it could be observed that the high-dose CpdA failed to suppress the TNBS-induced apoptosis. Instead an increased number of apoptotic cells were detected compared with untreated controls. Whereas low-dose CpdA (1 mg/kg) treatment of TNBS colitis only occasionally showed TUNEL-positive cells, in cross-sections of mice receiving high-dose CpdA (10 mg/kg) apoptosis was clearly prominent. Treatment of acute TNBS colitis with ZK216348 at both concentrations revealed low numbers of apoptotic cells comparable with control mice (Fig. 8, B and C). Accordingly, Western blot analysis showed an increase of the apoptotic markers caspase-8 and PARP-1 in tissue samples of mice 3 days post-TNBS that was most prominent in CpdA (10 mg/kg)-treated mice with TNBS colitis (Fig. 8B). Although the in vivo effect of CpdA alone was not examined, the apparently paradoxical failure of high-dose CpdA treatment is consistent with the in vitro proapoptotic effects of CpdA. The fact that at physiological pH the aziridine precursor CpdA is able to degrade into aziridines (Louw et al., 2000b; Wüst et al., 2009), known as highly reactive alkylating agents, would provide a rationale for the observed toxicity at higher CpdA doses, where aziridine concentration might exceed a critical threshold and cause unwanted effects such as apoptosis.

Discussion

Supported by a number of studies, it is assumed that SEGRAs act as selective GR modulators and exert their beneficial anti-inflammatory activities through the GR-mediated mechanism of transcriptional transrepression largely uncoupled from GRs’ transactivation mechanism, the latter being involved in the adverse effects of GCs (Robertson et al., 2010). Thus selective GR agonists have been suggested for the treatment of inflammatory disorders such as IBD with-
out facing GC therapy-associated complications. Investigations with the GR agonists CpdA and ZK216348 in a model of acute colitis demonstrated that the therapeutic effect of both substances substantially protected mice against the development of signs and symptoms of colonic inflammation and caused a reduction of the severity of colitis. These beneficial effects, observed for both doses of ZK216348 and a lower, subtoxic dose of CpdA, occurred in the absence of induction of GRE-driven gene expression and some classic steroid side effects.

Remarkably, whereas the administration of ZK216348 in our study showed a dose-dependent improvement of colitis symptoms, CpdA at the higher dose of 10 mg/kg surprisingly failed to improve the majority of colon inflammation parameters. Clearly this represents a sharp contrast to the predominant in vivo data of CpdA (De Bosscher et al., 2005; Dewint et al., 2008; Zhang et al., 2009; van Loo et al., 2010), showing that CpdA was able to prevent the disease-associated signs of inflammation in concentrations comparable with those used in this work. Yet, one study (Wüst et al., 2009) confirmed our observations of severe effects on the physiological condition of mice when CpdA was administered at higher concentrations for the treatment of experimental autoimmune encephalomyelitis. Consistent with our data those authors found lower doses of CpdA efficiently repressed disease symptoms in a GR-dependent manner, whereas administration of CpdA at 15 mg/kg was consistently lethal for C5Bl/6 mice. Supporting their finding with 1H-NMR spectroscopy, these effects were credited to the rapidly occurring decay of CpdA in buffered solutions, pH 7.6. At these conditions aziridine derivative formation takes place, a substance class known for its alkylating properties. Thus, if CpdA is applied at high concentrations, the presence of significant amounts of aziridine derivatives might account for the poor outcome in the treatment of experimental disease (Wüst et al., 2009). Similar to that publication and in contrast to ZK216348, with increasing concentrations of CpdA we could confirm a substantial induction of apoptosis by CpdA in human PBMCs in vitro. Although we conducted no in vivo experiments with high-dose CpdA administration alone, subsequent investigations into apoptosis in the acute TNBS colitis model revealed a lack of apoptosis prevention at CpdA 10 mg/kg, which could be attributed to the proapoptotic capacity of formed breakdown products of CpdA.
Despite the failure of the expected dose-response behavior of the GR modulator CpdA, both SEGRAs exhibited a superior profile regarding potential GC-associated side effects. In this study, we observed CpdA, at least at the low dose, and ZK216348 exert anti-inflammatory activities in vivo by down-modulating the inflammatory response, leading to a recovery of normal colon homeostasis. In addition to clinical and histological amelioration of TNBS colitis, CpdA and ZK216348 reduced key parameters of the Th1 immune response. Although neither a significant inhibition nor promotion of Th2-attributed cytokine release could be shown, increased expression of the Th2-relevant transcription factor GATA3 correlated with the disease outcome of colitis in mice. In chronic diseases the adaptive immune system and especially the defect in their CD4+ T cell-mediated and subsequent Th cell-mediated immune response, plays an important role in disease onset and progression (Strober et al., 2007). In the case of IBD, dysregulation of the Th1/Th2-type cytokine balance can lead to infiltration and destruction of otherwise healthy tissues, such as the gastrointestinal tract (Neurath et al., 2002). The prevention of excessive inflammation by means of the suppression of Th1 activation and the promotion of Th2 immune response (MacDermott, 1996) under therapy with SEGRAs may contribute to the resolution of IBD.

The positive regulation of gene transcription (transactivation) induces the synthesis not only of anti-inflammatory genes, but also of regulatory genes that are important for metabolism and hence involved in the side effects of GCs. Indeed, in transgenic animals with a defect in the dimerization domain of the GR (GR<sup>dim/dim</sup>), classic GRE-driven genes are not up-regulated after Dex administration (Reichardt et al., 1998b), and mice fail to present the GC treatment-associated side effect of increased gluconeogenesis. Of note in our experiments, the administration of Dex had an impact on glucose homeostasis, whereas treatment with CpdA and ZK216348 caused no response. Translated to therapy with selective GR modulators this could circumvent hyperglycemia and decrease the risk of development of “steroid diabetes.” Furthermore, our experiments revealed a considerable

Fig. 7. Effects of Dex or SEGRA treatment on GR expression and secretion of proinflammatory mediators. A and B, Western blot analysis and densitometric evaluation of total GR expression in MM6 cells (A) and human PBMCs (B) treated for 6 to 72 h with Dex or SEGRAs. C, determination of IL-6, IL-8, and MCP-1 secretion in cell culture supernatants of Dex- or SEGRA-pretreated and LPS (1 µg/ml)-stimulated PBMCs. Results show mean ± S.E.M. of three independent experiments. *<i>p</i> < 0.05; **<i>p</i> < 0.01; ***<i>p</i> < 0.001 versus vehicle or LPS treatment.
decrease in adrenocorticotropic hormone levels after treating mice with Dex, which was absent when administering low-dose CpdA. Therefore, severe conditions associated with HPA hypoactivation, e.g., adrenal insufficiency (Fuqua et al., 2010), could be avoided under SEGRA therapy. Treatment with ZK216348, on the other hand, mimicked the effects seen for Dex. This is in line with the results documented by Schäcke et al. (2004), who failed to detect clear advantages for ZK216348 against prednisolone with regard to HPA suppression. In the context of circulating corticosterone levels and their regulation of the HPA axis, it needs to be mentioned that from previous in vitro and in vivo studies CpdA was found to displace endogenous steroids from plasma corticosteroid-binding globulin. This, in turn, resulted in an increase of free corticosterone and a decrease of adrenocorticotropic hormone and corticosteroid-binding globulin levels (Louw and Swart, 1999; Louw et al., 2000b). Apart from illustrating another piece of the complex nature of CpdA, this finding stresses the point that the pharmacokinetics of individual SEGRAs may not be comparable. In the case of CpdA, an increase in free corticosterone leads to an increased risk for the appearance of GR-mediated side effects, thus at the same time neutralizing the contemplated advantage of selective GR agonists.

IBDs are chronic immune-mediated gut disorders, and in patients steroid hormone-related side effects usually appear after long-term treatment. Therefore, another major drawback and frequently observed side effect of GC therapy is a characteristic steroid resistance, caused in part by a down-modulation of GR expression in infiltrated cells and inflamed tissues (Barnes and Adcock, 2009). In our study Dex repressed GR expression in the human monocytic cell line MM6 and PBMCs, whereas CpdA and ZK216348 showed no such effect on receptor status. As a result, the potency of Dex to suppress the secretion of proinflammatory cytokines after “long-term” treatment was abrogated, which was not the case for CpdA or ZK216348. Thus, the risk of development of some side effects could be decreased under therapy with SEGRAs also during long-term treatment.

Herein, we demonstrated a definite beneficial therapeutic effect for ZK216348 and CpdA at least at lower doses in vivo, but at the same time we had to deal with profound toxicity at higher concentrations of CpdA. The narrow therapeutic window of CpdA has been defined (this study; Dewint et al., 2008; Wüst et al., 2009) and inevitably excludes CpdA from therapeutic application for any disease. Changes in the structure of CpdA might eradicate the aziridine formation-attributed toxicity and possibly increase the value of the low-molecular substance CpdA, although it is not clear whether it then still binds to and beneficially modulates GR action. More generally, even though in vitro and in vivo investigations of potential selective GR agonists, with the deployment of GR antagonists, might indi...
cating strong GR binding affinity and dissociation of GR-mediated beneficial of some side effects, CpdA is a good example that extrapolating to the in vivo situation is still risky. The possibility of other targets or substance-related unwanted effects of these GR agonists in vivo cannot be excluded. A detailed description of any SEGRA pharmacology to date is still missing, especially with respect to the structural diversity of these new GR agonists, and translation of the SEGRA concept into clinical benefit is still needed.

It is noteworthy that, contrary to CpdA, ZK216348 acted in the TNBS model in a dose-dependent manner, without induction of apoptosis or other unwanted side effects. This suggests that the toxicity observed with CpdA is not characteristic for the entire class of SEGRAs but rather due to CpdAs complex pharmacology. Possible specific differences between the diverse SEGRA substances are also underlined by the fact that ZK216348, which in addition to high affinity for GR, binds PR and MR in an antagonistic mode (Schäcke et al., 2004), has been shown to be a helpful investigational tool. Consequent further development led to the discovery of ZK245186, the first SEGRA in clinical testing (Schäcke et al., 2009).

Considering that GRs are present in many different cell types, organs, and tissues and play a role in diverse physiological responses, it is essential to more carefully characterize SEGRAs’ pharmacology and properties to find the appropriate candidate, which as a ligand, then properly elicits a beneficial activation/repression action of the GR in vivo. Nevertheless, SEGRAs or more precisely the concept of selective modulation of GR action toward predominantly gene suppression instead of activation still has great potential in the treatment of IBD and after optimization and characterization SEGRAs could become a valuable class of future therapeutics.

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