Effects of MCP-1 Inhibition by Bindarit Therapy in a Rat Model of Polycystic Kidney Disease

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Key Words

Polycystic kidney disease · PCK rat · Interstitial inflammation · MCP-1 · Bindarit · Proteinuria · Podocyte

Abstract

Background/Aims: Experimental and clinical evidence suggested that monocyte chemoattractant protein-1 (MCP-1/CCL2) has a role in the development of interstitial inflammation and renal failure in polycystic kidney disease (PKD). We investigated whether bindarit, an inhibitor of MCP-1/CCL2 synthesis, could influence the evolution of PKD in PCK rats. **Methods:** PCK rats were treated from 5 to 15 weeks of age with vehicle or bindarit. Sprague-Dawley rats served as control. For in vitro studies, murine podocytes were exposed to albumin with or without bindarit. **Results:** MCP-1 mRNA was upregulated in the kidney of PCK rats and reduced by bindarit. Treatment limited overexpression of MCP-1 protein by epithelial cells of dilated tubules and cysts, and interstitial inflammatory cells. Excessive renal accumulation of monocytes/macrophages was lowered by bindarit by 41%. Serum creatinine slightly increased in PCK rats on vehicle and was similar to controls after bindarit. Kidney and liver cysts were

not affected by treatment. Bindarit significantly reduced progressive proteinuria of PCK rats. The antiproteinuric effect was associated with the restoration of the defective nephrin expression in podocytes of PCK rats. Bindarit limited podocyte foot process effacement and ameliorated slit diaphragm frequency. In cultured podocytes, bindarit reduced MCP-1 production in response to albumin and inhibited albumin-induced cytoskeletal remodeling and cell migration. **Conclusion:** This study showed that although bindarit did not prevent renal cyst growth, it limited interstitial inflammation and renal dysfunction and reduced proteinuria in PKD. Thus, bindarit could be considered a therapeutic intervention complementary to therapies specifically acting to block renal cyst growth.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive PKD (ARPKD) are important causes of end-stage renal failure in adults and children [1, 2]. ADPKD and ARPKD are caused by muta-

tions in the *Pkd1/Pkd2* and *Pkhd1* genes respectively, that encode for polycystin (PC)-1/PC-2 and fibrocystin. Mutation or absence of these proteins may result in altered cell signaling with excessive tubular epithelial proliferation and fluid secretion, abnormal interactions of mutated epithelial cells with extracellular matrix, and alternatively activated interstitial macrophages that contribute to cyst formation [3]. There is no treatment to currently prevent renal failure due to PKD. The identification of signaling pathways and cellular changes underlying PKD has suggested candidate drugs to directly inhibit development or growth of the cysts, some of which are being tested in clinical trials [3–6]. There is growing awareness that interstitial inflammation is an important determinant of cystic renal disease [7]. Interstitial infiltrates of macrophages have been consistently documented both in human and experimental PKD [8–10], and monocyte chemoattractant protein-1 (MCP-1)/CCL2 was suggested to be implicated in the inflammatory cell recruitment. Thus, in a rodent model of PKD, the heterozygous Hannover-Sprague Dawley (Han:SPRD) rats, monocytes accumulated within the renal interstitium in parallel with increased levels of MCP-1/CCL2 in cystic mural cells and increased excretion in the urine [11]. High urinary levels of MCP-1/CCL2 were measured in ADPKD patients in association with the worsening of renal function [12].

In the present study, we evaluated whether bindarit, an inhibitor of MCP-1/CCL2 synthesis [13], could influence the evolution of PKD in the PCK rat model [14]. Subsequently, by *in vitro* experiments in cultured podocytes we investigated possible mechanisms underlying the effect of bindarit of limiting proteinuria and podocyte damage as found here in PCK rats.

Concise Methods

Experimental Animals

Animal care and treatment were in accordance with the institutional guidelines in compliance with national and international laws and policies [15]. Animal studies were approved by the Institutional Animal Care and Use Committee of IRCCS-Istituto di Ricerche Farmacologiche Mario Negri. Male PCK and Sprague-Dawley (SD) rats (Charles River Laboratories Italia, Calco, Italy) were kept on a 12-hour light/dark cycle with free access to standard diet and water. PCK rats were daily treated by gavage from 5 to 15 weeks of age with vehicle (0.5% methyl-cellulose) ($n = 10$), or bindarit (2-methyl-2-[[1-(phenylmethyl)-1H-indazol-3-yl] methoxy] propanoic acid), Angelini ACRAF S. Palomba-Pomezia, Italy, (100 mg/kg bid) ($n = 9$). Age-matched SD rats ($n = 8$) were used as control. Additional PCK and SD rats ($n = 5$ /each group) were sacrificed at 5 weeks of age for baseline kidney measurements.

Serum creatinine was measured by QuantiChrom™ Creatinine Assay Kit DICT-500 Gentaur, Brussels, Belgium. Proteinuria was measured by the Coomassie method.

Renal Histology and Cyst Morphometry

Duboscq-Brazil fixed, paraffin-embedded kidney sections (3 μ m) were stained with hematoxylin/eosin and periodic-acid Schiff reagent. Tubular casts were counted in at least 25 random fields ($\times 400$) for each animal. All renal biopsies were analyzed by the same pathologist who was unaware of the nature of the experimental groups.

For renal cyst analysis, sections were examined on a Zeiss light microscope (Zeiss, Jena, Germany) connected to a camera. For each kidney section, contiguous images were digitized using a 40 $\times$ objective. The fraction of area affected by cysts was estimated by point counting using a 26 $\times$ 19 line orthogonal grid digitally overlaid on the section images (ImageJ Software). For each section, the number of grid points hitting the cysts and those hitting the kidney tissue were counted. Fraction of kidney tissue volume affected by cysts was expressed as the percent ratio between grid points in the cysts area over total point in the kidney tissue.

Immunohistochemistry

MCP-1, WT-1, and nephrin were detected on paraffin-kidney sections using the immunoperoxidase technique. Microwave antigen retrieval in citrate buffer was performed before incubation with goat anti-MCP-1, rabbit anti-WT-1, or goat anti-nephrin primary antibodies (1:100, Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Biotinylated secondary antibodies were added, followed by avidin-biotin-peroxidase complex (ABC, Vector Lab, Burlingame, Calif., USA). The staining was visualized using diaminobenzidine, and sections were counterstained with Harris-type hematoxylin. Negative controls were obtained by omitting the primary antibody on adjacent sections. Mouse monoclonal antibody was used for the detection of monocyte/macrophage ED-1 surface antigen (1:100, Chemicon, Temecula, Calif., USA) by the alkaline phosphatase-Fast Red technique on paraffin-sections. Sections were counterstained with Harris-type hematoxylin. Positive-cells were counted in at least 30 glomeruli and 30 randomly selected interstitial high-power fields (HPF/ $\times 400$) for each animal.

Morphometric Analysis at Transmission Electron Microscopy (TEM)

Cortical kidney fragments were fixed in 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate for examination by TEM. Epithelial filtration slit frequency was evaluated on digitized TEM images and analyzed with the image processing software, Olympus iTEM analySIS. The glomerular basement membrane (GBM) profile was manually outlined using a line automatically measured in μ m. Slit frequency was evaluated as number of slits per μ m of GBM length.

Quantitative Real-Time PCR

Total RNA isolation from kidney and quantitative real-time PCR for MCP-1 was performed as described [15].

Liver Cysts

Formalin-fixed, paraffin-embedded liver sections (3 μ m) were stained with hematoxylin/eosin. Liver cysts were counted in at least 25 fields ($\times 200$).

Fig. 1. Bindarit limits renal MCP-1 upregulation in PCK rats. **a** MCP-1 mRNA expression was evaluated by RT-PCR in the kidney of PCK rats at 5 and 15 weeks of age. Bindarit treatment started at 5 weeks. Data are mean ± SE. * $p < 0.05$, ** $p < 0.01$ versus control; ° $p < 0.01$ versus vehicle. **b** MCP-1 protein expression was

detected in epithelial cells of dilated tubules and cysts and interstitial inflammatory cells in the kidney of PCK rats at 5 and 15 weeks of age. Magnification ×400. **c** MCP-1 protein was expressed in a few podocytes as indicated by staining for the podocyte marker WT-1 in adjacent sections (arrows). Magnification ×630.

In Vitro Studies

Immortalized mouse podocytes (obtained from Dr. Peter Mundel, Division of Nephrology, Massachusetts General Hospital, Charlestown, MA) were used. Differentiated podocytes were incubated with control medium or human serum albumin (HSA, Sigma Aldrich, Milan, Italy) (10 mg/ml) [16] with or without bindarit. MCP-1 production was measured by ELISA (Quantikine, R&D Systems, Minneapolis, Minn., USA). Albumin-induced F-actin cytoskeletal rearrangement was evaluated by fluorescence confocal microscopy [16]. Motility was evaluated in confluent podocytes scratched with a 200 µl-pipette to create a cell-free denuded area. Cells were exposed to albumin for different time intervals after scratch wound, with or without bindarit. Functional blocking anti-MCP-1 antibody (20 µg/ml, Abcam, Cambridge, UK) was used in selected experiments. Migration to scratched areas was assessed at time-lapse microscopy.

Statistical Analysis

Results are mean ± SE. Data were analyzed using ANOVA followed by Tukey's test, or non-parametric Mann-Whitney test. Statistical significance was defined as $p < 0.05$.

Results

MCP-1 Gene and Protein Expression in the Kidney of PCK Rats

The PCK rat is a model of polycystic kidney and liver disease developed spontaneously in the rat strain Crj:CD/SD. Although its pattern of inheritance is autosomal

recessive, it has many features that resemble human ADPKD [14]. First, we verified whether PCK rats were a suitable model to study the effects of MCP-1 inhibition by bindarit in the progression of PKD. We found that MCP-1 mRNA levels were already enhanced in the kidney of PCK rats at 5 weeks of age and further increased at 15 weeks as compared with matched-age control rats (fig. 1a). MCP-1 gene upregulation was paralleled by excessive expression of protein mainly localized to epithelial cells of dilated tubules and cysts and to interstitial inflammatory cells (fig. 1b). At the glomerular level, few podocytes were also positive for MCP-1 (fig. 1c). When PCK rats were treated with bindarit from 5 to 15 weeks of age, both MCP-1 mRNA and protein were reduced with respect to PCK rats receiving vehicle (fig. 1a, b).

Renal Accumulation of Inflammatory Cells is Reduced by Bindarit

An increased number of ED-1 positive monocytes/macrophages was found in the kidney of PCK rats given vehicle as compared to control rats (fig. 2). Inflammatory cells mainly infiltrated the renal interstitium, but were also detected in the glomeruli. MCP-1 inhibition by bindarit therapy resulted in a significant reduction of both glomerular and interstitial accumulation of monocytes/macrophages (fig. 2).

Fig. 2. Renal accumulation of inflammatory cells is reduced by bindarit. ED-1 positive monocytes/macrophages were evaluated in glomeruli and renal interstitium of PCK rats at 5 and 15 weeks of age. Positive cells were counted in at least 30 glomeruli and 30 randomly selected interstitial high-power fields (HPF/X400) for each animal. Data are mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus control; ° $p < 0.05$, °° $p < 0.01$ versus vehicle. Representative images show ED-1 positive monocytes/macrophages (arrows) in the kidney of SD control rats, PCK rats given vehicle or bindarit at 15 weeks of age. Magnification $\times 400$.

Effect of Bindarit on Renal Function and Proteinuria in PCK Rats

Serum creatinine was slightly increased in PCK rats on vehicle at 15 weeks (0.72 ± 0.03 mg/dl versus controls: 0.54 ± 0.02 mg/dl, $p < 0.05$). After bindarit, values (0.64 ± 0.05 mg/dl) were not different from controls. In PCK rats given vehicle, proteinuria progressively increased with respect to control rats, with values being significant within 13 weeks of age (fig. 3a). Treatment with bindarit resulted in a remarkable reduction of proteinuria.

Histological analysis of the kidneys revealed the presence of tubular casts in PCK rats on vehicle (fig. 3b). Consistent with the reduced protein filtration in response to bindarit, less tubular casts were counted in bindarit-treated PCK rats when compared to the vehicle group.

Bindarit Preserves Podocyte Structure of PCK Rats

On the basis of the antiproteinuric effect observed in bindarit-treated PCK rats, we investigated for possible drug's effects on the podocyte structure. We evaluated the expression of nephrin, a slit diaphragm protein that functions to maintain slit pore integrity and renal filtration capacity. An intense signal of nephrin with the typical epithelial-like staining pattern was detected in glomeruli of control rats. PCK rats exhibited a significant reduction in nephrin expression, which was almost restored by bindarit treatment (fig. 4). Glomerular ultrastructure

analysis in PCK rats showed hyaline droplets in podocytes and focal foot process effacement (fig. 5a). These changes were less severe in the glomeruli of bindarit-treated rats. A remarkable reduction in the frequency of filtration slit pores was measured in PCK rats on vehicle compared with controls (fig. 5b). After bindarit, slit pore frequency was significantly increased.

Kidney and Liver Cysts

The relative kidney weight in PCK rats was higher than in the control rats (table 1). No changes were observed after bindarit. Quantification of the kidney cysts indicated that cyst growth of PCK rats was not reduced by bindarit. Abnormal liver weight and cysts in PCK rats were not affected by bindarit treatment (table 1).

In Vitro Studies

To investigate mechanisms underlying the antiproteinuric effects observed in bindarit-treated PCK rats, in vitro experiments were performed in differentiated murine podocytes exposed to albumin overload. Albumin induced a time-dependent release of MCP-1/CCL2 that was significantly reduced by the addition of bindarit (fig. 6a), with a dose-dependent effect (fig. 6b). Reorganization of podocyte cytoskeleton in response to albumin [16], as shown by redistribution of F-actin fibers to the periphery of the cells (fig. 7), was almost prevented by

Fig. 3. Antiproteinuric effect of bindarit in PCK rats. **a** Time course of urinary protein excretion in control rats, PCK rats treated with vehicle or bindarit. **b** Tubular casts were analyzed in kidney sections stained with periodic acid-Schiff and counted in high power fields of interstitial areas (HPF/X400). Data are mean $\pm$ SE. * $p < 0.01$ versus control; ° $p < 0.01$ versus vehicle. Magnification $\times 200$.

Fig. 4. Defective nephrin expression in podocytes of PCK rats is ameliorated by bindarit. Nephrin expression was evaluated in glomeruli of control rats, PCK rats treated with vehicle or bindarit at 15 weeks of age by immunoperoxidase technique. Intensity of

nephrin signal was graded on a scale of 0 to 3 (0, absent; 1, weak; 2, moderate; 3, strong staining) in 30 glomeruli on average per each animal. Data are mean $\pm$ SE. * $p < 0.01$ versus control; ° $p < 0.01$ versus vehicle. Magnification $\times 1,000$.

Fig. 5. Bindarit limits podocyte damage in PCK rats. **a** Glomerular ultrastructural changes in PCK rats given vehicle as evaluated by transmission electron microscopy (TEM) at 15 weeks of age showed hyaline droplets in podocytes (asterisk), focal foot process effacement (arrows), and thickening of the basement membrane (inset). Magnification $\times 7,100$. **b** Morphometric analysis of slit diaphragm frequency was analyzed in three animals per group. An

average of 50 images per animals was digitized (Magnification $\times 56,000$) and analyzed with the image processing software, Olympus iTEM analySIS. The glomerular basement membrane (GBM) profile was manually outlined using a line automatically measured in μm . Slit frequency was evaluated as number of slits per μm of GBM length. Data are mean $\pm$ SE. * $p < 0.01$ versus control; ° $p < 0.05$ versus vehicle.

Table 1. Body weight, kidney, and liver weight and cysts in PCK and control rats

Groups	Body weight (g)	Kidney weight (%BW)	Kidney cysts (%)	Liver weight (%BW)	Liver cysts (no. cysts/field 20 $\times$)
PCK					
Vehicle	503 $\pm$ 10	1.5 $\pm$ 0.2**	26 $\pm$ 2.3**	6.0 $\pm$ 0.7*	8.8 $\pm$ 0.7**
Bindarit	483 $\pm$ 10	1.7 $\pm$ 0.1**	29 $\pm$ 1.6**	5.8 $\pm$ 0.5	10.3 $\pm$ 1**
Control	514 $\pm$ 12	0.7 $\pm$ 0.02	2.6 $\pm$ 0.6	3.9 $\pm$ 0.1	0 $\pm$ 0

Values are expressed as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ vs. control.

Fig. 6. Effect of bindarit on MCP-1 production in response to albumin in cultured podocytes. **a** Cells were incubated with medium alone or human serum albumin (HSA, 10 mg/ml) for 6, 15, 24, and 48 h, in the presence or absence of bindarit (600 μ M). **b** Podocytes were incubated with medium alone or HSA for 24 h,

in the presence or absence of bindarit (100, 300, 600 μ M). MCP-1 production was measured in cell supernatants by ELISA and normalized to total protein content. Data are expressed as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus control; ° $p < 0.05$, °° $p < 0.01$ versus HSA.

Fig. 7. Effect of bindarit on F-actin rearrangement induced by albumin in cultured podocytes. **a–c** Representative images of immunofluorescence staining for F-actin fibers (red) and ZO-1 (green), a marker that defines cell periphery, in podocytes exposed for 6 hours to medium alone (**a**), HSA 10 mg/ml (**b**), HSA plus bindarit 600 μ M (**c**). Nuclei were labelled by 4',6-diamidino-2-phenylindole (DAPI, blue). Cells with F-actin rearrangement are signed with asterisks. Magnification $\times 630$, scale bars = 10 μ m. **d** Quantification of podocytes with F-actin remodeling. Data (mean $\pm$ SE) are expressed as percentage of cells with F-actin rearrangement with respect to total cells ($n = 5$ fields for each sample; $n = 3$ experiments). * $p < 0.01$ versus control; ° $p < 0.05$ versus HSA.

Fig. 8. Effect of bindarit on albumin-mediated podocyte migration. Podocyte motility was evaluated by scratch healing assay at time lapse microscopy for 24 h. Podocytes were exposed to HSA (10 mg/ml) with or without bindarit (600 μ M) or neutralizing anti-MCP-1 antibody (20 μ g/ml). The number of migrated cells into a uniform-size field was counted at each time point. ** $p < 0.01$ versus control; ° $p < 0.05$, °° $p < 0.01$ versus HSA.

MCP-1/CCL2 inhibition by bindarit. The effect of bindarit on podocyte motility in response to albumin was evaluated using the scratch healing assay. Albumin induced podocyte migration when compared to unstimulated control cells with a maximum effect at 24 h. Bindarit maintained the number of migrated podocytes to values similar to control (fig. 8). A functional blocking anti-MCP-1 antibody reduced albumin-induced podocyte migration, but to a lesser extent than bindarit (fig. 8). These data indicate that albumin promoted podocyte migration mainly via MCP-1/CCL2. However, since bindarit besides MCP-1/CCL2 also inhibits the synthesis of MCP-3/CCL7 and MCP-2/CCL8 [13] we cannot exclude an involvement of all these chemokines in albumin-mediated migration of podocytes.

Discussion

Bindarit is a selective inhibitor of the monocyte chemotactic protein subfamily of CC inflammatory chemokines (MCP-1/CCL2, MCP-3/CCL7, MCP-2/CCL8) [13], acting through the down-regulation of NF- κ B pathway [17]. Bindarit has been proven to exert anti-

inflammatory activity in a number of experimental diseases, including arthritis and pancreatitis [18]. In murine lupus nephritis, bindarit reduced MCP-1 mRNA overexpression and signs of inflammation in the kidney, delayed the development of proteinuria and renal damage, and prolonged survival [19]. Moreover, in patients with acute proliferative lupus nephritis, bindarit significantly reduced albuminuria and urinary MCP-1 levels [20].

Here we show that bindarit limited MCP-1/CCL2 up-regulation in the kidney of PCK rats and that inhibition of the chemotactic signal translated in a reduced accumulation of inflammatory cells in the kidney. These data while confirming potent anti-inflammatory effects of bindarit in renal disease [19] support the notion that MCP-1/CCL2 is a chief instigator of inflammatory cell recruitment that plays an important role in PKD [11, 12]. In Han:SPRD rats, increases in MCP-1 mRNA coincided with increased numbers of CD68-positive macrophages and correlated with the development of renal failure [11]. Data in *pck* mice a model of recessive PKD, showed that among genes overexpressed in most severely affected kidneys, were those associated with activated macrophage markers [21]. Moreover, there is also in vi-

tro evidence that *Pkd1*^{-/-} tubular cells expressed significantly higher levels of MCP-1 mRNA than *Pkd1*^{f/f} cells [9]. The anti-inflammatory effect of bindarit in PCK rats was associated with normalization of renal function, albeit mildly impaired at 15 weeks of age in PCK rats given vehicle. Bindarit was not able to reduce the number of cysts in the kidney. The apparent lack of effects of bindarit on cyst growth could possibly be explained by the fact that bindarit did not completely abrogate macrophage accumulation in the renal interstitium. Thus, residual macrophages at cystic area could still contribute to cyst growth [9]. It is not excluded that higher doses of bindarit could have provided more inhibitory effects on macrophage accumulation and consequently on cystogenesis. Moreover, besides macrophages, it has to be considered that there are several diverse pathophysiologic mechanisms including dysregulation of intracellular calcium levels and cAMP signaling that mediate cyst growth [3].

An interesting finding of the present study is the significant antiproteinuric effect afforded by bindarit in PCK rats. Although proteinuria is not a common finding in PKD, its presence identifies a subset of patients with more severe renal involvement both structurally and functionally [22]. Microalbuminuria and proteinuria have been found to correlate with the progression of renal disease in ADPKD [22, 23]. Bindarit ameliorated podocyte damage of PCK rats as indicated by restoration of defective nephrin expression and the presence of less foot process effacement in association with increased slit pore frequency when compared to untreated rats, which underlined proteinuria lowering effect by bindarit. Here, in vitro studies in murine podocytes exposed to albumin overload were instrumental to establish that amelioration of podocyte structure and

antiproteinuric effect by bindarit in PCK rats could be ascribed to drug's ability of inhibiting podocyte MCP-1/CCL2 production and its deleterious effects on these cells. It has been, in fact, reported that upon binding to the podocyte's CCR2 receptor, MCP-1/CCL2 could increase cellular motility, cause actin cytoskeleton rearrangement, and increase podocyte permeability to albumin [24]. Moreover, in cultured human podocytes MCP-1 binding to CCR2 induced reduction of both nephrin mRNA and protein expression [25]. Our data consistently indicated that bindarit by inhibiting MCP-1/CCL2 overproduction caused by albumin exposure counteracted podocyte dysfunction limiting cytoskeletal changes and podocyte motility. The effects of the drug on podocyte phenotypic changes in response to albumin possibly reflects in vivo condition in which bindarit may influence cell-cell interaction and cell matrix adhesion to GBM, thereby limiting podocyte detachment and disruption of glomerular filtration barrier.

In conclusion, although bindarit did not prevent renal cyst growth, it reduced proteinuria and limited interstitial inflammation in PKD. Thus, bindarit could be considered a therapeutic intervention complementary to future therapies specifically acting to block renal cyst growth.

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Disclosure Statement

Angelo Guglielmotti is an employee of Angelini ACRAF.

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