Original Research

Effect of dimethyl fumarate on renal disease progression in a genetic ortholog of nephronophthisis

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Impact statement

This is the first study to investigate the effects of dimethyl fumarate in a model of cystic kidney disease. The study assessed the therapeutic efficacy of dimethyl fumarate in upregulating renal nuclear factor erythroid-derived factor 2 expression, reducing macrophage accumulation and cyst progression in a Lewis polycystic kidney disease rat model. This study demonstrates that dimethyl fumarate significantly upregulated renal nuclear factor erythroid-derived factor 2 expression and attenuates renal macrophage infiltration, but had no effect on renal cyst progression, cardiac enlargement, and improving renal function.

Abstract

Dimethyl fumarate is an FDA-approved oral immunomodulatory drug with anti-inflammatory properties that induces the upregulation of the anti-oxidant transcription factor, nuclear factor erythroid-derived factor 2. The aim of this study was to determine the efficacy of dimethyl fumarate on interstitial inflammation and renal cyst growth in a preclinical model of nephronophthisis. Four-week-old female Lewis polycystic kidney disease (a genetic ortholog of human nephronophthisis-9) rats received vehicle (V), 10 mg/kg (D10) or 30 mg/kg (D30) ($n = 8$–9 each) dimethyl fumarate in drinking water for eight weeks. Age-matched Lewis control rats were also studied ($n = 4$ each). Nuclear factor erythroid-derived factor 2 was quantified by whole-slide image analysis of kidney sections. Renal nuclear factor erythroid-derived factor 2 activation was partially reduced in vehicle-treated Lewis polycystic kidney disease rats compared to Lewis control (21.4 ± 1.7 vs. $27.0 \pm 1.6\%$, mean $\pm$ SD; $P < 0.01$). Dimethyl fumarate upregulated nuclear factor erythroid-derived factor 2 in both Lewis Polycystic Kidney Disease (D10: 35.9 ± 3.8; D30: $33.6 \pm 3.4\%$) and Lewis rats (D30: $34.4 \pm 1.3\%$) compared to vehicle-treated rats ($P < 0.05$). Dimethyl fumarate significantly reduced CD68+ cell accumulation in Lewis polycystic kidney disease rats (V: 31.7 ± 2.4; D10: 23.0 ± 1.1; D30: 21.5 ± 1.9; $P < 0.05$). In Lewis polycystic kidney disease rats, dimethyl fumarate did not alter the progression of kidney enlargement (V: 6.4 ± 1.6; D10: 6.9 ± 1.2; D30: $7.3 \pm 1.3\%$) and the percentage cystic index (V: 59.1 ± 2.7; D10: 55.7 ± 3.5; D30: $58.4 \pm 2.9\%$). Renal dysfunction, as determined by the serum creatinine (Lewis + V: 26 ± 4 vs. LPK + V: 60 ± 25 $P < 0.01$; LPK + D10: 47 ± 7; LPK + D30: 47 ± 9 μmol/L), and proteinuria were also unaffected by dimethyl fumarate treatment. In conclusion, the upregulation of nuclear factor erythroid-derived factor 2 by dimethyl fumarate reduced renal macrophage infiltration in nephronophthisis without adverse effects, suggesting that it could potentially be used in combination with other therapies that reduce the rate of renal cyst growth.

Keywords: Dimethyl fumarate, nuclear factor erythroid-derived factor 2, Lewis polycystic kidney disease rats, macrophage, nephronophthisis

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Introduction

Juvenile familial nephronopthsis (NPHP) are a heterogeneous group of multi-systemic and progressive autosomal recessive cystic kidney diseases characterized by the growth of cortio-medullary cysts and tubulointerstitial inflammation.[1,2] It is a rare disease with an incidence of 1:50,000 with 20 causative genes (*NPHP1–20*) but is the most common genetic cause of end-stage kidney disease in the first three decades of life.[2] Despite the heterogeneity, the mutated genes encode proteins localized to cilia which lead to increased renal epithelial cell proliferation and loss of differentiation, resulting in the formation of kidney cysts

and tubulointerstitial disease.[3–5] Due to advances in the understanding of the genetics and molecular pathophysiology, new drugs to treat cystic kidney diseases are anticipated to emerge.[6] In particular, the post-natal growth of renal cysts is, in part, dependent on arginine vasopressin (AVP) and treatment with an AVP receptor antagonist has been shown to reduce disease progression in a preclinical model of NPHP.[7] However, AVP receptor antagonists may not be tolerated by all patients with NPHP due to off-target effects,[8] and other drugs need to be investigated.

Tubulointerstitial inflammation is a key histopathological feature of NPHP and other cystic kidney diseases.[9–12] The expansion of fluid-filled cysts in the renal microenvironment elicits immunological alterations, evinced by the infiltration of macrophages and neovascularization.[9,10] Recent studies have provided evidence that macrophage accumulation and cyst progression are positively correlated.[10,13,14] Thus, reducing macrophage load could serve as an important intervention in NPHP, as proposed by Slaats *et al*.[6] Many pathways could be targeted to reduce interstitial inflammation. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor involved in regulating the expression of antioxidant proteins such as catalase and heme-oxygenase 1 to protect against oxidative damage and modulate inflammation.[15] While previous studies have shown the inhibition of Nrf2 in animal models of chronic kidney disease (CKD) increases inflammation and oxidative stress, its status in NPHP remains unknown.[16,17] However, based on the similarities in the histopathological features of CKD, it is plausible that Nrf2 signaling is altered in NPHP. Therefore, a pharmacological intervention activating the Nrf2 pathway could potentially be beneficial in the management of NPHP.

At present, there are numerous immunomodulatory drugs which could alleviate interstitial inflammation and slow renal cyst progression.[18–20] DMF is the methyl ester of fumaric acid. DMF is known to activate the Nrf2 pathway and inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) NF-κB can in turn decrease Nrf2 transcription and activity, and several studies have suggested that NF-κB activation is upregulated in NPHP, and may be associated with increased inflammatory mediators.[21,22] Following oral administration, DMF is hydrolyzed by esterases into monomethylfumarate (half-life = 56 min), the active metabolite.[23] DMF has a high bioavailability with peak serum levels (mean 6 μM, range 3–10 μM) observed at $\asymp$178 min after a 120 mg oral DMF administration, given to healthy individuals.[23] DMF has a good pharmacokinetics profile with no evidence of cytochrome P450-dependent metabolism.[24] In addition, it has excellent long-term tolerability, with mild side effects (flushing, gastrointestinal events and proteinuria) reported in only a minority of patients, and no increased risk for infection.[25,26] DMF is currently approved for the treatment of multiple sclerosis and other inflammatory disorders, marketed in Australia under the trade name Tecfidera.[27,28]

The aim of the study was to investigate the impact of DMF on renal Nrf2 expression, macrophage accumulation, and cyst progression using an animal model of NPHP – the Lewis polycystic kidney (LPK) rat. Based on the pharmacology of DMF, we hypothesized that DMF would upregulate renal Nrf2 expression, reduce macrophage accumulation, slow renal cyst progression, and thus could be a potential drug therapy for NPHP.

Methods

Lewis polycystic kidney disease rats as a model of NPHP

The LPK rat is a genetic ortholog of human nephronopthsis-9 which is caused by a spontaneous mutation of *Nek8*.[29] Phenotypic characteristics include cystic dilatation of the distal tubules and collecting ducts (progressing significantly between week 6 and 10), tubulointerstitial disease, hypertension, and ultimately the development of end-stage renal disease after week 20.[30] In this study, female LPK rats and Lewis rats were acquired from the breeding colony at Westmead Hospital. Rats were housed under standard conditions (artificial lighting; light: dark cycle 1800–0600 h) at the Animal Care Department at Westmead Hospital and allowed food and water ad libitum.[31] All protocols and procedures were approved by the Western Sydney Local Health District Animal Ethics Committee (Protocol Number 4100), and conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes.[32]

Experimental design

Four-week-old female LPK rats received either vehicle (water) (V) ($n = 9$), 10 mg/kg (D10) ($n = 8$) or 30 mg/kg (D30) ($n = 9$) DMF dissolved in drinking water for eight weeks. Similarly, age-matched Lewis rats were administered either the vehicle (V) ($n = 4$) or 30 mg/kg DMF (D30) ($n = 4$). Urine was collected by placing animals in metabolic cages at weeks 8 and 12. At the endpoint of the study (week 12), rats were anaesthetized by an intraperitoneal injection of ketamine and xylazine, and a mid-line laparotomy was performed. Blood was obtained by cardiac puncture, and the heart and kidneys were removed, weighed, and immediately placed in fixation solution.

DMF was purchased from Sigma-Aldrich (CA, USA), prepared as a stock solution and stored at −20°C. The route of DMF administration and doses was determined based on a previous study by Casili *et al.*,[33] which showed that doses of 10 and 30 mg/kg DMF were safe and demonstrated significant anti-inflammatory potential. The DMF stock concentration was determined based on a previous pilot study which showed that Lewis and LPK rats, on average, weigh 200 g and consume ~20 ml of water per day. The stock solution (600 mg/L in water) was given to the D30 group and subsequently diluted (300 mg/L in water) for the D10 group. Water bottles were weighed every one to three days to monitor the amount of water, and consequently DMF, consumed by each rat.

Immunohistochemistry and histology

Mid-coronal kidney sections were fixed in either 10% formaldehyde or methyl Carnoy's solution for 12 h, then paraffin embedded. For histological analysis, 4–6 μm thick sections were deparaffinized and then either stained with periodic acid-Schiff (PAS) or Sirius red. For immunohistochemistry, deparaffinization, peroxide inactivation (0.03 g/ml hydrogen peroxide), and antigen retrieval (microwave oven 100% power heating for 10 min in 1 × Antigen Decloaker) (Biocare Medical, CA) were performed based on a protocol described previously.[34] The primary antibodies: anti-Nrf2 (1:100, ab31163; Abcam, Cambridge, UK) and anti-CD68 (1:400, MCA341R; Serotec, Kidlington, UK) were used to assess renal Nrf2 expression and macrophage accumulation, respectively. Following overnight incubation with primary antibodies, secondary biotinylated antibodies were applied for 30 min at room temperature (anti-mouse, 1:200, 65–6440; anti-rabbit, Life Technologies, USA). Vectastain ABC reagent (Vector Laboratories, Burlingame, USA) was applied for 20 min, followed by diaminobenzidine.[34] Sections were counterstained with methyl green (Sigma-Aldrich, CA, USA) then dehydrated.[34] Following staining, the slides were scanned using the NanoZoomer slide scanner (Hamamatsu Photonics, Shizuoka, Japan).

Quantification of Nrf2 and ED-1+ cells was performed individually by whole-slide image analysis using Aperio ImageScope (v11.2.0.780, Leica Biosystems, USA). The kidney section was outlined using the pen tool. The standardized positive pixel count (version 9) algorithm was then applied to determine the presence of Nrf2 and ED-1+ cells, as indicated by the strong intensity staining of the kidney section. This value was then expressed as a percentage of the total kidney section area.

Assessment of kidney cyst progression and enlargement

Kidney cyst progression was assessed by the percentage two-kidneys to body weight ratio (KW:BW) and the percentage of kidney section occupied by the cyst (cystic index). Kidney and body weight were measured by electronic balance (Sartorius Co, Hamburg, Germany). The cystic index was calculated using Aperio Imagescope (v11.2.0.780, Leica Biosystems, USA). Firstly, a border around the kidney section was drawn using the pen tool, followed by the application of the positive pixel count (version 9) algorithm, which highlights the renal tissue only, excluding cysts. This area was then subtracted from the total area of the kidney section, surrounded by the border to obtain the area occupied by the cysts. The area was then expressed as a percentage of the total kidney section area to obtain the cystic index.

Assessment of renal function

To assess for urine creatinine and protein to creatinine ratio (Pr:Cr), urine was collected by placing rats in metabolic cages for 16 h. To prevent chronic stress, rats were not acclimatized in metabolic cages prior to urine collection. In addition, serum was analyzed for creatinine and urea. Urine and blood serum analysis were conducted by the Institute of Clinical Pathology and Medical Research. Creatinine clearance was then calculated using a previous method.[35]

Western blot

Protein extracts were obtained from 100 mg of kidney cortex from rats in each treatment group at week 12 ($n = 4$ per group). Kidney tissue was homogenized in cold radioimmunoprecipitation assay buffer (Cell Signaling), with enzyme inhibitors. Protein concentrations were assessed using the DC™ Protein Assay (Bio-Rad). Thirty micrograms of total protein was resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred into nitrocellulose membranes (Licor, Lincoln, NE). Western blot was done using a previously described method.[36] Blots were probed with NF-κB p105/p50 (1:1000, ab32360, Abcam) and β-actin (1:1000, #4970, Cell Signaling) and visualized on an Odyssey Imaging System (Licor). Densitometry was quantified using ImageJ (v1.47, National Institutes of Health, United States) and normalized using β-actin.

Statistical analysis

Results are presented as mean ± SD. Statistical analysis and graph construction were performed using JMP statistical software package (v4.04, SAS institute, USA) and GraphPad Prism (v6.04 for Windows, GraphPad Software, USA), respectively. The statistical test used for multivariate analysis was ANOVA, followed by the Tukey-Kramer HSD test for *post hoc* analysis. *P*-value less than 0.05 was considered as statistically significant.

Results

Effects of DMF on general health and body weight

There was no mortality or morbidity in any animals and DMF was tolerated with no adverse effects. The body weight of all groups increased in the early stage of the disease (week 4 to 7). From week 7, body weight increased more gradually than before, in all the experimental groups. There were no significant differences in mean body weight of the experimental groups over time (Figure 1(a) and (b)).

Consumption of oral DMF in the experimental groups

Water consumption increased over time as rats aged. LPK rats consumed more water than Lewis rats (Figure 2(a)) but there were no group-specific differences (that is, water consumption was similar in all LPK groups). The amount of water consumption reflected DMF ingested. After day 30, the DMF dose consumed by the three treatment groups was higher than 10 mg/kg and 30 mg/kg dose anticipated for the study animals (see Figure 2(b) and Methods).

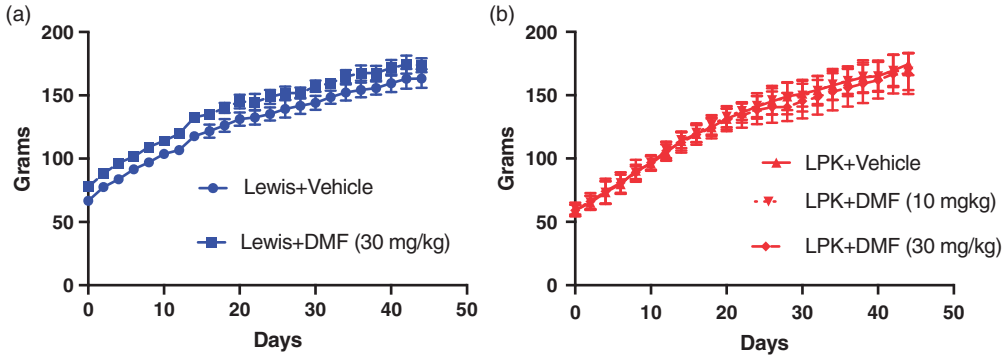

Figure 1. Effect of chronic DMF treatment on body weight in Lewis (Panel a; Blue lines) and LPK (Panel b, Red Lines) rats. Time-course of body weight of each experimental group from week 4 to week 12. No significant difference in mean body weight between experimental groups over time ($P = 0.78$). DMF: dimethyl fumarate; LPK: Lewis polycystic kidney disease. (A color version of this figure is available in the online journal.)

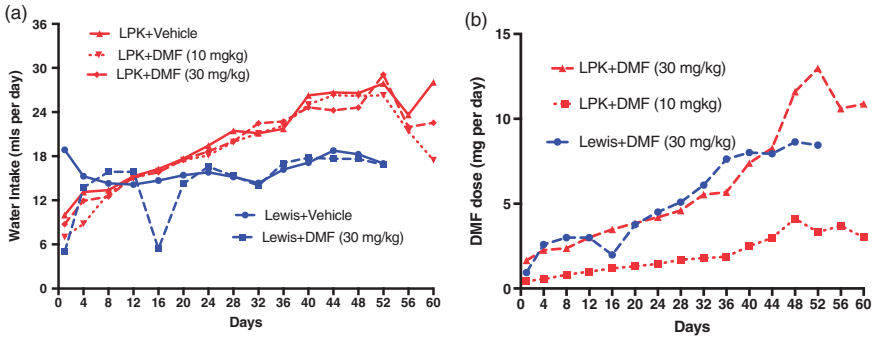

Figure 2. Mean daily water consumption per rat in each treatment group (a) and mean amount of daily DMF consumed per rat in each treatment group, including true 10 mg/kg and 30 mg/kg doses (b). The blue lines represent Lewis rats and the red lines represent LPK rats. DMF: dimethyl fumarate; LPK: Lewis polycystic kidney disease. (A color version of this figure is available in the online journal.)

Effect of DMF on renal expression of Nrf2 in Lewis and LPK rats

Renal Nrf2 expression was reduced in LPK rats compared to Lewis rats ($P < 0.01$) (Figure 3 and Table 1). Treatment with DMF increased renal Nrf2 expression in both Lewis and LPK rats (both $P < 0.01$) compared with control rats (Figure 3 and Table 1).

Effect of DMF on interstitial inflammation in Lewis and LPK rats

Renal macrophage accumulation, as assessed by CD68 immunohistochemistry increased in LPK compared to Lewis rats (Figure 3 and Table 1). Treatment with DMF reduced renal macrophage accumulation compared to untreated LPK rats (Figure 3 and Table 1).

Effects on kidney enlargement and kidney cyst growth in LPK rats

Kidney enlargement, as assessed by the KW:BW ratio, was approximately 8-fold higher in the LPK rats compared to the Lewis rats ($P < 0.01$) (Figure 4(a)). Treatment with DMF produced no significant change in KW:BW in both groups (Figure 4(a)). At week 12, there was diffuse cystic dilatation of the collecting ducts and distal tubules in the kidneys of LPK rats, but not Lewis rats. Quantitative histological analysis revealed that the cystic index of LPK rats was

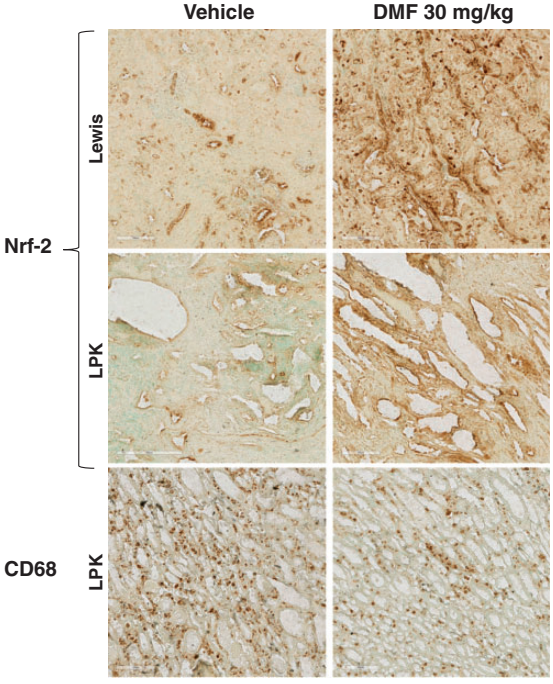

Figure 3. Effect of chronic DMF treatment on renal Nrf2 and CD68 expression as assessed by immunohistochemistry. Histological changes were analyzed in sections taken from kidney cortices at week 12 in Lewis and LPK rats in the various treatment groups (Scale bar = 100 μm). DMF: dimethyl fumarate; LPK: Lewis polycystic kidney disease. (A color version of this figure is available in the online journal.)

considerably higher relative to Lewis rats ($P < 0.01$) (Figures 4(a) and 5). Treatment of LPK and Lewis rats with DMF produced no significant change in cystic index compared to the respective vehicle-treated groups (Figure 5). Additionally, Sirius red staining indicated that there was no significant difference in interstitial fibrosis in any of the experimental groups.

Effect of DMF on renal dysfunction and proteinuria in LPK rats

The serum creatinine was ~2-fold higher in LPK rats than in Lewis rats ($P < 0.01$) (Table 2), but this was not altered by DMF treatment. The serum urea was ~3-fold higher in LPK rats than in Lewis rats ($P < 0.01$), and this was also not altered by DMF (Table 2). Additionally, the creatinine clearance was reduced in LPK rats relative to Lewis rats ($P < 0.01$), but again no significant change with DMF (Figure 6(a)). Proteinuria was also not affected by treatment with DMF (Figure 6(b)).

Effect of DMF on cardiovascular disease in LPK rats

Cardiac enlargement, as assessed by heart to body weight ratio (HW:BW) was approximately 3-fold higher in LPK rats than that of the Lewis rats ($P < 0.01$; Figure 4(b)). There was no significant change in HW:BW in LPK and Lewis rats treated with DMF (Figure 4(b)).

Effect of DMF on renal NF-κB expression

Western blotting of whole kidney homogenate found that NF-κB p50 expression was similar in all treatment groups, and DMF did not affect the expression of NF-κB p50

Table 1. Effects of chronic DMF treatment on Nrf2, CD68, and kidney cyst growth in the treatment groups at week 12.

Treatment group	Nrf2 (%)	CD68+ cells (%)	Cystic index (%)
Lewis+vehicle	26.9 ± 1.6	–	9.6 ± 3.4
Lewis+DMF (30 mg/kg)	34.4 ± 1.3#	–	12.7 ± 2.8
LPK+Vehicle	21.4 ± 1.7	31.7 ± 2.4	59.1 ± 2.7
LPK+DMF (10 mg/kg)	35.9 ± 3.8*	22.9 ± 1.1*	55.7 ± 3.5
LPK+DMF (30 mg/kg)	33.6 ± 3.4*	21.5 ± 1.9*	58.4 ± 2.9

#$P<0.05$ when compared to Lewis + Vehicle.
*$P < 0.05$ when compared to LPK + Vehicle.
Note: *n*: four per group for Lewis and *n*: eight to nine per group for LPK rats.
DMF: dimethyl fumarate; LPK: Lewis polycystic kidney disease.

Table 2. Renal function in Lewis and LPK rats at week 12.

Treatment group	Serum creatinine (μM)	Serum urea (mM)
Lewis+vehicle	26 ± 4*	6 ± 1*
Lewis+DMF (30 mg/kg)	27 ± 2*	5 ± 1*
LPK+Vehicle	60 ± 25	17 ± 6
LPK+DMF (10 mg/kg)	47 ± 7	16 ± 3
LPK+DMF (30 mg/kg)	46 ± 9	17 ± 3

Note: Data expressed as mean ± SD. *n*: four per group for Lewis rats and *n*: eight to nine per group for LPK rats.
*$P < 0.01$ when compared to LPK + Vehicle.
LPK: Lewis polycystic kidney disease.

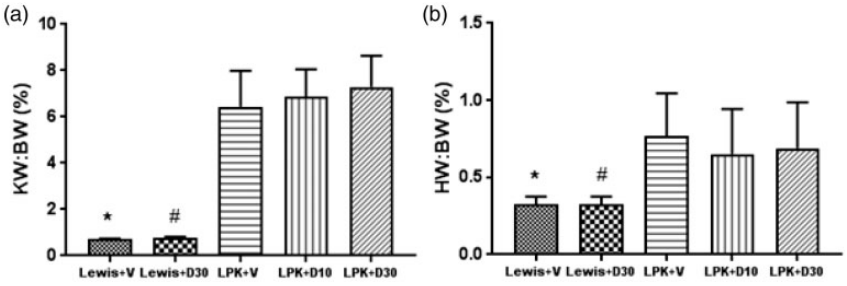

Figure 4. (a) Effect of chronic DMF treatment on the percentage kidney to body weight ratio at week 12. Data expressed as mean ± SD. *$P < 0.01$ when compared to LPK + V; #$P < 0.01$ when compared to LPK + D30. (b) Effect of DMF on the percentage heart weight to body weight ratio at week 12. Data expressed as mean ± SD. *$P < 0.01$ when compared to LPK + V; #$P < 0.01$ when compared to LPK + D30. $n = 4$ per group for Lewis rats and $n = 8$–9 per group for LPK rats. DMF: dimethyl fumarate; LPK: Lewis polycystic kidney disease.

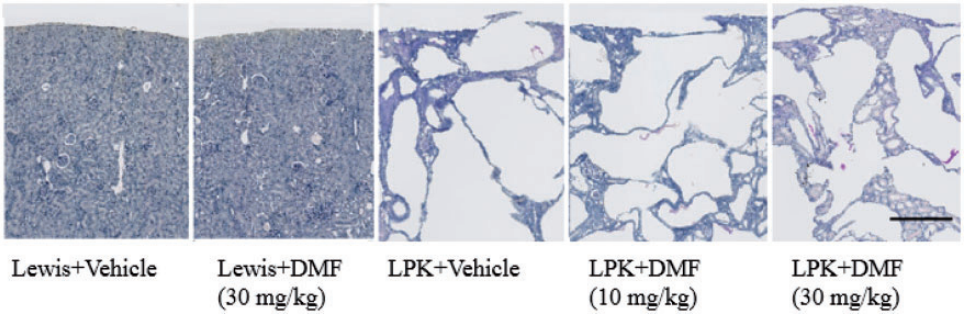

Figure 5. Effect of chronic DMF treatment on kidney cyst growth. Whole-slide digital images of periodic acid Schiff-stained kidney cortices of Lewis and LPK rats in various treatment groups at week 12. No difference in cyst area between LPK groups. Scale bar = 300 μm. DMF: dimethyl fumarate; LPK: Lewis polycystic kidney disease. (A color version of this figure is available in the online journal.)

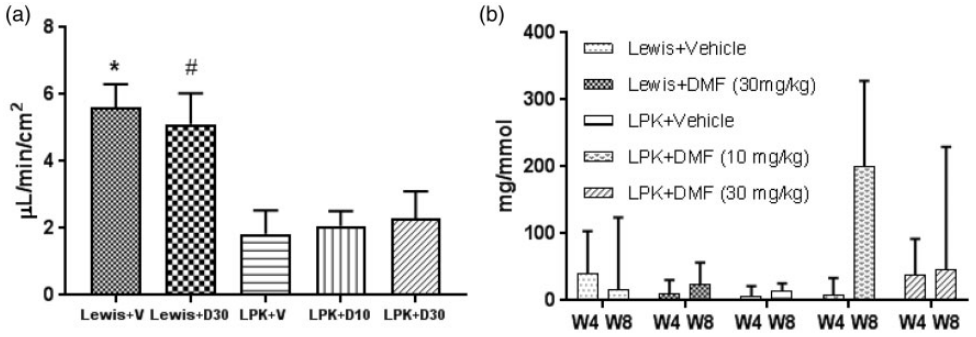

Figure 6. Effect of DMF on the endogenous creatinine clearance (week 8) (a) and proteinuria (week 4 and week 8) (b). DMF had no effect on improving creatinine clearance and proteinuria. Data expressed as mean ± SD; *$P < 0.01$ when compared to LPK + V; #$P < 0.01$ when compared to LPK + D30. $n = 4$ per group for Lewis rats and $n = 8$–9 per group for LPK rats. DMF: dimethyl fumarate; LPK: Lewis polycystic kidney disease.

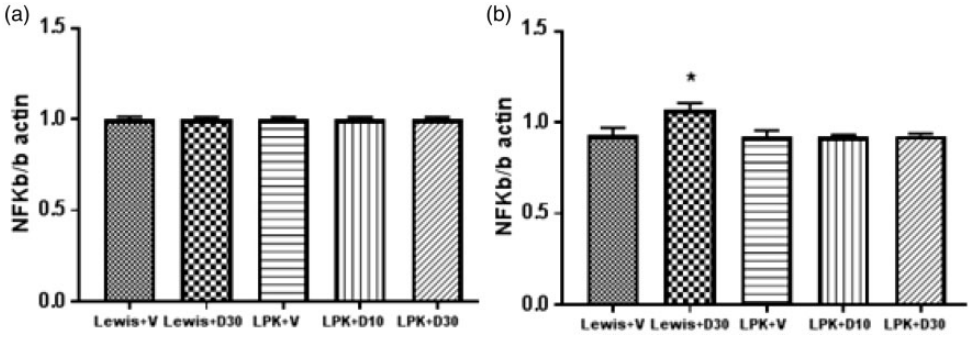

Figure 7. Effect of chronic DMF treatment on the whole kidney expression of NF-κB p50 and p105 proteins in Lewis and LPK rats, as assessed by Western blotting. Immunoblotting and quantification of densitometry were performed for (a) p50, and (b) p105 in all treatment groups ($n = 4$ per group). *$P < 0.01$ for p105 expression in the Lewis + D30 treatment group compared to other treatment groups. DMF: dimethyl fumarate; LPK: Lewis polycystic kidney disease.

(Figure 7(a)). However, p105 was mildly elevated in the Lewis + D30 treatment group compared to vehicle-treated Lewis animals ($P < 0.01$; Figure 7(b)).

Discussion

In the past decade, many studies have demonstrated that the extent of renal interstitial inflammation in cystic kidney diseases is positively correlated to renal cyst growth.[9–12] Research into drugs that target these pathways has thus increased substantially. However, there has been no investigation into the effect of DMF, an immunomodulatory drug already approved for the treatment of multiple sclerosis, in the management of NPHP or other cystic kidney diseases.[37]

This study assessed the therapeutic efficacy of DMF in reducing macrophage accumulation and cyst progression, and upregulating renal Nrf2 expression in a preclinical model of NPHP. The primary finding of the study was that the Nrf2 signaling pathway was impaired in LPK rats, as indicated by the lower renal Nrf2 expression detected in LPK rats as compared to Lewis rats. Following DMF treatment, renal Nrf2 expression in the distal tubules, was restored. This concurs with previous studies demonstrating activation of the Nrf2 pathway as a major mechanism of action of DMF.[38,39] Under homeostatic circumstances, the basal level of Nrf2 is determined by Kelch-like ECH-associated protein 1 (KEAP1), which facilitates the ubiquitination of Nrf2 by Cullin 3, and its subsequent degradation by proteasomes.[15] Pharmacological studies have shown that DMF modifies specific cysteine residues on KEAP1, thereby preventing Nrf2 ubiquitination and degradation.[21,40] This mechanism explains the significant increase in renal Nrf2 expression observed in our study. Interestingly, our results indicated that the effect of DMF on renal Nrf2 expression was not dose dependent, contrary to a study by Saidu *et al.*,[21] which observed increasing Nrf2 level with increments in DMF dose. This discrepancy could be attributed to different experimental protocols used in the respective studies.

Our study has also demonstrated that DMF reduced renal macrophage accumulation. We could partially attribute this reduction to the increase in renal Nrf2 expression produced by DMF. Nrf2 would translocate to the nuclear antioxidant response element (ARE) promoter, and ultimately result in an increase in antioxidant protein level. Kobayashi *et al.*[41] have shown that Nrf2 inhibits the transcription of pro-inflammatory cytokines responsible for the recruitment of macrophages to the renal interstitium. Through this mechanism, DMF treatment reduces macrophage accumulation and our results provide an inverse correlational relationship between Nrf2 and macrophage

accumulation. However, we found no significant difference in the expression of p50, the active subunit of NF-κB. In this study, the expression of p50 was similar in both Lewis and LPK rats, and was not affected by DMF.

Despite the reduction in interstitial inflammation seen with DMF treatment, cyst progression was not reduced, as determined by the cystic index and KW:BW was not halted. This suggested that the decline in interstitial inflammation produced by DMF was insufficient to cause cyst regression. To date, there is only a limited number of studies that have investigated the relationship between interstitial inflammation and cyst progression. Our data have similar findings to the study by Ta *et al.*,[42] which have documented unaltered percentage cyst area but significant reduction in KW:BW, concurrently with significant reduction in macrophage load following sirolimus administration. However, contrary to Wu *et al.*,[20] which found that a reduction in macrophage infiltration by ~7% in Male Han: SPRD (Cy/+) rats, following resveratrol administration, produced a substantial decline in cystic volume density and KW:BW. Similarly, Karihaloo *et al.*[14] showed significant improvements in KW:BW and cystic index in *Pkd1*$^{fl/fl}$; *Pkhd1-Cre* mice treated with liposomal clodronate (depletes macrophages by 95%). The discrepancy with our data could be accounted for by phenotypic and genotypic differences in the animal model, off-target effects by resveratrol and a considerably larger decline in macrophage load in the study by Karihaloo *et al.* The minimal number of preclinical studies on the effect of anti-inflammatory compounds on macrophage infiltration and cyst progression in PKD makes it difficult to deduce the degree of reduction in interstitial inflammation required for cyst progression to be halted.

The current study also demonstrated that DMF was ineffective in reducing cardiac manifestations of NPHP. Left ventricular hypertrophy commonly develops in PKD and is thought to occur due to hypertension.[30,43] It is now understood that hypertension in PKD is due to the release of renin from cystic epithelial cells, triggered by the compression of intra-renal micro-vessels by the expanding renal cysts.[30,44] Since DMF had no effect on inhibiting kidney cyst progression in LPK rats, hypertension can progress and ultimately augment HW:BW.

We also failed to see significant improvement in renal function in LPK-treated rats. This could be explained by continued cyst progression. Cyst progression compresses the surrounding renal parenchyma, and progressively compromises renal function. Our results agree with the study by Gattone *et al.*,[18] which demonstrated a direct relationship between cyst regression, and serum urea nitrogen in cystic male Han:SPRD (Cy/+), following methylprednisolone administration. However, our results contradict Serra *et al.*,[19] who showed a delayed decline in renal function regardless of increasing cyst volume in humans following sirolimus administration. The discrepancy could be attributed to difference in the pathogenesis of PKD in animal models and humans, in addition to the off-target effects of sirolimus. We also showed a trend towards reduced serum creatinine in DMF-treated LPK rats relative to untreated LPK rats, despite similarity in serum urea, creatinine clearance, and Pr:Cr. This highlights the complex relationship between cyst progression and renal function, which is determined by other factors such as interstitial inflammation and fibrosis.[45]

Despite being ineffective in reducing cyst progression, renal function and cardiac enlargement, DMF was effective in reducing interstitial inflammation. In addition, our results indicated that DMF has a good toxicity profile, with no potential adverse effects as suggested by unchanged KW:BW, HW:BW, and renal function parameters. This concurs with two large phase 3 clinical trials which showed no significant difference in the most commonly reported adverse effects (dyspepsia and flushing) between the DMF-treated group (240 mg) and placebo.[46] The maximal tolerated dose of DMF in animals and humans is unclear. In addition, the potential for a dose-dependent effect on cyst progression has not been established. Optimization of DMF delivery and renal concentration of the active metabolite may further aid our understanding of whether a higher dose of DMF could further reduce inflammation and cyst progression. In addition, while there are numerous anti-inflammatory compounds in the market, a few such as resveratrol and sirolimus are poorly understood in terms of its pharmacology and are associated with adverse side effects (respectively).[19,20] With similar anti-inflammatory potential, as previous studies have shown,[47–49] DMF with its minimal side effects and relatively high efficacy is a potential alternative for the management of PKD.

Although the present study was undertaken in a genetic ortholog of NPHP, future work should investigate its role in PKD and/or PKHD1 knockout rats (orthologous to human ADPKD and/or ARPKD respectively) to bridge our understanding of the potential effect of DMF in other cystic kidney diseases. In addition, since this study did not distinguish between different macrophage subtypes, and only the M2-like macrophage subtype has been implicated in cyst progression, future studies should aim to identify the M2 subtype to determine whether it is affected by DMF administration. Pro-inflammatory cytokines and chemokines such as TNF-α, IL-6, and MCP-1 elaborated by inflammatory infiltrates are thought to be involved in cyst progression, and should be quantified in future studies.[11,12]

In conclusion, DMF was effective in reducing renal interstitial inflammation, but not renal cyst progression and renal impairment in a genetic ortholog of NPHP. Future directions should combine DMF with other drugs that definitively cause cyst regression, to determine possible synergistic effects outcomes in . These include drugs such as tolvaptan, a vasopressin receptor 2 antagonist and octreotide, a somatostatin analog. Both significantly slow the increase in total kidney volume in clinical trials.[8,50] Furthermore, studying the impact of regulated water intake and dietary modifications – such as low sodium intake – in conjunction with DMF could also aid in our understanding as to whether a combination treatment affects cyst regression.[51,52]

Authors' contributions: All authors (OO, PR, ML, PS, CM, AW, NR and GR) participated in the design, interpretation of the

studies and analysis of the data, and review of the manuscript. PR performed the animal studies and tissue collection. OO wrote majority of the manuscript, analyzed and interpreted data. ML, PS and NR conducted the Western blot, analyzed and interpreted data. AW and CM reviewed and interpreted data and approved the final version of the manuscript. GR designed the experiments, performed animal studies, oversaw all experiments, reviewed all data and reviewed the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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