Dexamethasone Prodrug Treatment Prevents Nephritis in Lupus-prone (NZB×NZW)F1 Mice without Causing Systemic Side Effects

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Abstract

Objective—To evaluate the potentially improved therapeutic efficacy and safety of nephrotropic macromolecular prodrugs of glucocorticoids (GC) in the treatment of lupus nephritis.

Methods—Monthly injection of N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-based dexamethasone prodrug (P-Dex) and daily injection of dexamethasone phosphate sodium (Dex, overall dose equivalent to P-Dex) were given to lupus-prone (NZB×NZW)F1 female mice for two months. The animals were monitored for albuminuria, mean arterial pressure and serum autoantibody levels during the treatment. Nephritis, renal immune complexes and macrophage infiltration were evaluated histologically. The bone quality was analyzed with pDEXA and μ-CT. Optical imaging, immunohistochemistry (IHC) and fluorescence-activated cell sorting (FACS) were used to understand the in vivo distribution of P-Dex. The anti-inflammatory effect of P-Dex was validated using LPS-activated human proximal tubule epithelial cells (HK-2).

Results—Monthly P-Dex injection completely abolished albuminuria in the (NZB×NZW)F1 mice, which is significantly (P < 0.001) more efficacious than daily Dex treatment. P-Dex did not reduce serum levels of anti-dsDNA antibodies or renal immune complexes, but did reduce macrophage infiltration, a marker of chronic inflammation. IHC and FACS analyses revealed that P-Dex was primarily sequestered by proximal tubule epithelial cells and it could attenuate the inflammatory response in HK-2 cell culture. Different from Dex treatment, P-Dex did not lead to any significant bone quality deterioration or total serum IgG reduction.

Conclusion—Macromolecularization of GCs renders them nephrotropic. The protracted retention, subcellular processing and activation of GC prodrugs by kidney cells would potentiate nephritis resolution with reduced risk of systemic toxicities.

Lupus is an autoimmune disease in which autoantibodies are produced against nuclear antigens, including double stranded DNA (dsDNA). Renal deposition of anti-dsDNA IgG containing immune complexes leads to nephritis, a major cause of morbidity and mortality in lupus patients. Renal immune complexes induce inflammation and immune cell...
infiltration, which if unresolved, lead to renal injury, dysfunction, and failure. Nephritis is treated with glucocorticoids (GCs), which are suboptimal because they frequently cause off-target toxicity. Because lupus patients often take GCs continuously for many years, they are at high risk for developing GC-associated adverse side effects, including osteoporosis and immunosuppression.

The therapeutic efficacy of a drug depends on its specificity for its molecular target and its concentration at the site of interaction with the target. Advances in understanding lupus have stimulated progress in the identification of drugs that interact with molecular targets and pathways associated with disease [1]. These efforts, nevertheless, have not addressed the problems created by our inability to control the in vivo drug concentration at either the intended site(s) of action or off target sites, where drug action results in adverse side effects.

To address this challenge, we have developed a macromolecular prodrug of dexamethasone (P-Dex), which passively targets inflamed tissues and provides superior and sustained resolution of inflammation in several animal models [2–4]. Here, we demonstrate that P-Dex prevents nephritis in lupus-prone (NZB×NZW)F1 mice. P-Dex demonstrated reduced systemic toxicity compared to the equivalent dose of dexamethasone. Mechanistic studies indicate that the nephrotropism, cell-mediated local sequestration, subcellular processing and activation of P-Dex likely contribute to its superior therapeutic efficacy and reduced systemic toxicities.

**MATERIALS AND METHODS**

**Synthesis of macromolecular prodrugs**

P-Dex (Figure 1A) was synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization as described previously [3]. Briefly, N-(2-hydroxypropyl)methacrylamide (HPMA), N-methacryloylglycylglycylhydrazyl dexamethasone (MA-Dex) [3] and other comonomers {N-methacryloylaminopropyl fluorescein thiourea [5] and N-(3-aminopropyl)methacrylamide hydrochloride (APMA, Polysciences, Inc. Warrington, PA)} were copolymerized at 40°C under Argon for 48 hours (h) with 2,2′-azobisisobutyronitrile as the initiator and S,S′-bis(α, α′-dimethyl-α”-acetic acid) trithiocarbonate as the RAFT agent [6]. The resulting polymers were purified by LH-20 column (GE HealthCare, Waukesha, WI) and lyophilized. IRDye 800CW and Alexa Fluor® 488 labeled P-Dex (P-Dex-IRDye and P-Dex-Alexa) were obtained via polymer analogous reactions between poly(HPMA-co-MA-Dex-co-APMA) and NHS esters of these dyes [2].

**Experimental animals and drug treatment**

(NZB×NZW)F1 and NZW females (Jackson Laboratories, Bar Harbor, ME) were housed under controlled humidity, temperature and lighting conditions in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, operating in accordance with standards set by the Guide for the Care and Use of Laboratory Animals (The National Academies Press, 1996). Mice were given Harlan irradiated rodent diet 7904 (Harlan Teklad, Madison, WI) and allowed to feed ad libitum. All procedures involving live animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

At 16 weeks of age, mice were treated via i.v. injection with saline, N-(2-hydroxypropyl)methacrylamide homopolymer (PHPMA), or P-Dex (250 mg/kg) every 4 weeks. A fourth group of mice were given daily i.p. injections of dexamethasone 21-phosphate disodium (Dex, 1.32 mg/kg, containing 1.00 mg/kg of dexamethasone, Hawkins,
Dosages of Dex and P-Dex were calculated and prepared as such that mice received the same dose of dexamethasone over the 8-week treatment period.

Mice were monitored weekly for albuminuria using Albustix (Siemens Corp., Washington DC). Albuminuria was defined as two consecutive 2+ readings (100 mg/dL). Every 4 weeks, serum was isolated from peripheral blood, and mean arterial pressure (MAP) was recorded via tail-cuff method using the CODA blood pressure measuring system and software (Kent Scientific, Torrington, CT). One week after cessation of treatment, mice were euthanized and tissues harvested.

**Analysis of nephritis, renal immune complexes and macrophage infiltration**

Kidneys were fixed, paraffin-embedded, sectioned and stained with Periodic Acid-Schiff (PAS) (Sigma-Aldrich, St. Louis, MO) and analyzed by light microscopy. One hundred glomeruli per mouse were evaluated as described previously [7]. Renal immune complexes were visualized by immunohistochemistry. After deparaffinization and rehydration, slides were incubated in H2O2, washed and incubated in citrate buffer (Vector Laboratories, Burlingame, CA). Slides were blocked with normal horse serum (Vector Labs), and incubated with anti-mouse IgG (Vector Labs). Antibody binding was visualized using Vectastain Elite reagents (Vector Labs). Staining intensity (represented as arbitrary gray units or AGU) of fifty glomeruli per mouse was quantified using Axiovision software (v4.6.3.0; Carl Zeiss, Thornwood, NY). A second set of slides stained for immune complexes was counterstained with hematoxylin; these slides were for illustration purposes only.

Renal macrophage infiltration was assessed via immunofluorescence with the macrophage marker Iba-1 (Biocare Medical, Concord, CA) as described previously [8], with an added blocking step in Sudan Black B. Staining was visualized and quantitated using confocal microscopy and Zen 2010 software (v6; Carl Zeiss).

**Serological analysis of serum immunoglobulin levels**

Serum immunoglobulin concentrations were determined by ELISA (Southern Biotech, Birmingham, AL). The IgG1, IgG2a, IgG2b, and IgG3 levels were added together to obtain total serum IgG levels. Serum Anti-dsDNA IgG levels were determined by ELISA (Alpha Diagnostics International, San Antonio, TX) as described previously [7].

**Analysis of bone quality**

Femoral bone mineral density (BMD) and micro-architectural parameters were measured as described previously [9], using pDEXA® Sabre™ X-ray bone densitometer (Norland Medical System, Inc, Fort Atkinson, WI) and Skyscan 1172 micro-CT system (Skyscan, Kontich, Belgium), respectively. pDEXA analysis was performed with a 20 mm/sec scanning speed and a 0.2 × 0.2 mm resolution. Areal BMD was calculated using SABRE RESEARCH software (v3.9.4). Micro-CT scanning parameters were: voltage, 55 kV; current, 189 μA; exposure time, 230 ms; resolution, 6.2 μm; and aluminum filter (0.5 mm). Three-dimensional reconstructions were performed with NRecon and Dataviewer software (Skyscan). Trabecular bone was selected for analysis by a polygonal region of interest within the center of the femur, starting at 20 slices (0.25 mm) proximal from the growth plate and extending proximally 80 slices (0.99 mm) further. Trabecular bone volume fraction, number and thickness were quantified with CTAn software (Skyscan).
Near infrared imaging analysis

Mice received P-Dex-IRDye (148 nmol IRDye per kg body wt.) by i.v. injection. Mice were euthanized 2 or 7 days (d) later and tissues were harvested and imaged using an XENOGEN IVIS® 200 Series Imaging System (Caliper Life Sciences, Hopkinton, MA).

Immunohistochemical analyses of P-Dex distribution within kidney

Mice were given P-Dex-Alexa (300 nmol Alexa Fluor® 488 per kg body wt.) via i.v. injection. Seven days later, mice were perfused and euthanized. Kidneys were fixed, paraffin-embedded and sectioned. For direct staining, APC-labeled anti-mouse B220 and CD8α, and PE-labeled anti-mouse CD31 and CD4 (BD Pharmingen, San Diego, CA) antibodies were used. For indirect staining, sections were incubated with anti-mouse CD11c (eBioscience, San Diego, CA), E-cadherin (R&D Systems, Minneapolis, MN), F4/80 and Ly-6G (eBioscience) antibodies, followed with PE-labeled secondary antibody (eBioscience; R&D Systems; and Invitrogen, Carlsbad, CA). Stained sections were examined under a Nikon Swept Field confocal microscope (Nikon Instruments Inc, Melville, NY).

Flow cytometry

Mice received an i.v. injection of P-Dex-Alexa. At necropsy (7 d post injection), white blood cells (WBCs) were isolated from peripheral blood. Mice were perfused, and tissues were isolated, macerated and passed through a 70-μm strainer to obtain single cell suspensions. Cells were analyzed with Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The following antibodies were used: APC-labeled anti-mouse B220, CD19, CD5 and CD8a; PE-labeled anti-mouse F4/80 (eBioscience), Ly6B.2 (AbD Serotec, Raleigh, NC), CD4, CD138, CD31 and IgD (BD Pharmingen); PECy7-labeled anti-mouse IgM (BD Pharmingen); anti-mouse CD11c, α-smooth muscle actin and E-cadherin followed with PE-labeled secondary antibody.

Cell culture

Human proximal tubule epithelial cells (HK-2) were grown in RMPI 1640 with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (0.1 mg/mL). To investigate the internalization of fluorescein isothiocyanate (FITC) labeled P-Dex (P-Dex-FITC), HK-2 cells were stimulated overnight with LPS (10 μg/mL). P-Dex-FITC (final concentration 200 μg/mL) was added to LPS-stimulated and untreated HK-2 cells. After specified intervals, cells were rinsed and analyzed by FACS.

For subcellular localization studies, HK-2 cells were cultured overnight with LPS and then incubated with P-Dex-FITC (200 μg/mL) for 24 h. Cells were rinsed and incubated with 75 nM Lysotracker DND-99 (Invitrogen) for 3 h. After rinsing, cells were stained with DAPI, fixed, mounted and observed by confocal microscopy.

To analyze the anti-inflammatory effect of P-Dex, HK-2 cells were treated with LPS plus Dex (2 μM) or P-Dex (2 μM dexamethasone equivalent) for 24 h. Supernatants were collected and stored at −80°C. Cells were rinsed and incubated with fresh medium containing LPS for an additional 48 h. Supernatants were collected and assayed for IL-6 level by ELISA (R&D Systems).

Statistical methods

Comparisons were performed using Fishers exact test, Wilcoxon signed-ranks test, Mann-Whitney U test, independent or paired samples t-test, or one-way ANOVA with Tukey’s post hoc test where appropriate. Statistical analyses were performed using SPSS software.
A two-sided $P \leq 0.05$ was considered significant. Two-sided p-values are provided. Mean ± standard error of the mean is presented.

RESULTS

P-Dex prevents albuminuria and reduces glomerular damage

Albuminuria was measured in (NZB×NZW)F1 mice to assess nephritis-associated loss of renal function. Prior to treatment, none of the mice displayed albuminuria. However, after 8 weeks, 100% of saline treated mice and 70% of PHPMA treated mice exhibited albuminuria (Figure 1B). The incidence of albuminuria in these groups did not differ significantly ($P = 0.2$). After eight weeks, 47% of Dex treated mice displayed albuminuria (Figure 1B), which was significantly different from the saline ($P < 0.01$), but not the PHPMA group ($P = 0.4$). Strikingly, after eight weeks, 0% of P-Dex treated mice exhibited albuminuria (Figure 1B), which is significantly different from the saline ($P < 5 \times 10^{-7}$), PHPMA ($P < 5 \times 10^{-3}$) and Dex ($P < 5 \times 10^{-2}$) groups. Thus, P-Dex was more effective than Dex in preventing albuminuria.

To further assess renal function, PAS-stained kidney sections were analyzed for glomerular abnormalities induced by nephritis. Abnormal glomeruli were found at a frequency of 16% in the saline group and 14.9% in the PHPMA group (Figure 1C, 1D). There was no significant difference between these two groups ($P = 0.9$). The frequency of abnormal glomeruli in Dex and P-Dex treated mice was 11.3% and 9.9%, respectively (Figure 1C, 1D). There was no significant difference between the Dex and P-Dex groups ($P = 0.7$), but the frequency in both groups was significantly lower than that in the saline group ($P < 0.01$). Although the frequency of abnormal glomeruli in the Dex and P-Dex groups was lower than that in the PHPMA group, the difference achieved significance for the P-Dex ($P < 5 \times 10^{-3}$) but not the Dex ($P = 0.07$) group. Thus, both Dex and P-Dex preserve the structural integrity of glomeruli, suggesting that these treatments attenuate nephritis.

P-Dex does not reduce anti-dsDNA IgG levels or renal immune complexes

Nephritis in (NZB×NZW)F1 mice correlates with serum levels of pathogenic anti-dsDNA IgG [10]. Therefore, serum anti-dsDNA IgG levels were assessed. Over the 8-week time course, serum anti-dsDNA IgG levels increased in the saline ($P = 0.07$) and PHPMA ($P < 0.01$) groups, although this increase fell short of statistical significance in the saline group (Figure 2A). Over this time period, serum anti-dsDNA IgG levels rose significantly in the Dex and P-Dex groups (Figure 2A; $P \leq 0.01$), indicating that neither treatment prevented this pathognomonic increase in anti-dsDNA IgG. Nonetheless, at the 8-week time point, serum anti-dsDNA IgG levels in the Dex group were significantly lower than those in the PHPMA and P-Dex groups ($P < 0.05$). Thus, Dex blunts the increase in anti-dsDNA IgG, which correlated with reduced incidence of albuminuria and nephritis in this group. By contrast, in the P-Dex group, reduced glomerular damage did not correlate with serum anti-dsDNA IgG, suggesting that P-Dex prevents nephritis through a mechanism that is independent of production of pathogenic autoantibodies.

Because nephritis in (NZB×NZW)F1 mice is associated with renal deposition of anti-dsDNA IgG-containing immune complexes [11], renal immune complex deposition was evaluated (Figure 2B). Quantification of staining indicated that immune complex deposition in the PHPMA treated group did not differ from that in the saline group (Figure 2C; $P \geq 0.2$). Renal immune complex staining in the Dex treated group was significantly less than that in the other groups (Figure 2C; $P < 1 \times 10^{-12}$). Immune complex staining in the P-Dex group was not reduced compared to saline or PHPMA groups (Figure 2C; $P \geq 0.7$). Thus, in contrast to Dex, P-Dex does not prevent nephritis by diminishing renal immune complex deposition.
**P-Dex reduces renal macrophage infiltration**

To test the hypothesis that P-Dex reduced renal inflammation, we examined renal macrophage infiltration, a marker of chronic inflammation. Staining with the macrophage marker Iba-1 was detected in all groups. Quantification of Iba-1 staining indicated that macrophage infiltration in the saline and PHPMA groups did not differ (Figure 3; *P* = 0.4). Macrophage infiltration was not significantly reduced in the Dex group compared to either the saline (*P* = 0.2) or PHPMA (*P* = 0.7) group (Figure 3). By contrast, renal macrophage infiltration was significantly lower in the P-Dex group than in the saline and PHPMA groups (Figure 3; *P* < 0.04). Although macrophage infiltration was less abundant in the P-Dex group than the Dex group, this difference fell just short of statistical significance (Figure 3; *P* = 0.06). These results suggest that P-Dex may maintain renal function by attenuating renal inflammation.

**P-Dex reduces mean arterial pressure**

Because GC therapy can lead to hypertension, we assessed the impact of each treatment on blood pressure. In mice treated with either saline or PHPMA, MAP was not significantly altered after 8 weeks (Figure 4A; *P* ≥ 0.4). In the Dex group, MAP was reduced after 8 weeks of treatment (Figure 4A), although this decrease fell short of statistical significance (*P* = 0.08). By contrast, P-Dex significantly reduced MAP after 8 weeks of treatment (Figure 4A; *P* < 1×10\(^{-4}\)).

**P-Dex treatment does not affect bone quality**

Long-term GC use is associated with osteoporosis. To understand the impact of P-Dex on the skeleton, the femoral BMD and micro-architecture were evaluated. No significant difference in femoral BMD was observed between the saline and PHPMA groups (Figure 4B). Dex treatment, however, was associated with a significantly lower BMD (Figure 4B; *P* < 5×10\(^{-7}\)). By contrast, BMD in the P-Dex group did not differ from that in the saline and PHPMA groups (*P* > 0.05). No significant differences in trabecular bone volume fraction or number were found between groups. However, in the Dex group, trabecular thickness was significantly lower than that in the other groups (Figure 4C; *P* < 0.05). Thus, in contrast to free Dex, P-Dex did not negatively affect BMD or microarchitecture of the bone.

**P-Dex treatment does not reduce serum IgG levels**

GC therapy causes immunosuppression and reduces serum IgG [12, 13]. We therefore analyzed the impact of treatment on serum immunoglobulin levels. None of the treatments affected serum IgA or IgM levels (data not shown). Prior to treatment, no significant differences in serum IgG levels were observed between groups (Figure 4D; *P* > 0.4). In the saline and PHPMA groups, serum IgG concentrations increased significantly after 8 weeks (Figure 4D; *P* = 0.01). In the Dex group, serum IgG level decreased significantly after 8 weeks (Figure 4D; *P* < 5×10\(^{-3}\)). By contrast, serum IgG concentration increased after 8 weeks in the P-Dex group (Figure 4D; *P* < 5×10\(^{-3}\)). At the 8-week time point, Dex treated mice had significantly lower serum IgG levels than mice in the saline, PHPMA, and P-Dex groups (*P* < 5×10\(^{-3}\)). Thus, in contrast to free Dex, P-Dex did not reduce serum IgG.

**P-Dex exhibits nephrotropism in (NZB×NZW)F1 mice**

To elucidate the mechanism underlying the enhanced efficacy and decreased toxicity of P-Dex in (NZB×NZW)F1 mice, optical imaging was performed to evaluate the in vivo distribution of P-Dex. (NZB×NZW)F1 and NZW (healthy control) mice received i.v. injections of P-Dex-IRDye and P-Dex-Alexa and imaging was performed at 2 and 7 d post injection. P-Dex-IRDye preferentially accumulates (2 d) and is retained (7 d) in inflamed kidneys of (NZB×NZW)F1 mice, but not healthy kidneys of NZW controls (Figure 5A).
FACS revealed that ~61% of kidney cells from (NZB×NZW)F1 mice were P-Dex-Alexa+ whereas less than 20% of kidney cells from NZW mice were P-Dex-Alexa+ (Figure 5B). Furthermore, the mean fluorescence intensity of P-Dex-Alexa+ kidney cells of (NZB×NZW)F1 mice was substantially greater (~4.5 fold) than that of P-Dex-Alexa+ kidney cells in NZW mice. This observation suggests that on a per cell basis, larger quantities of P-Dex are taken up and retained in the kidneys of (NZB×NZW)F1 mice than NZW mice. Persistent near-infrared fluorescence signals were also observed in the spleen and liver of (NZB×NZW)F1 mice (Figure 5A). FACS analysis showed that ~81% of spleen cells and ~10% of liver cells were P-Dex-Alexa+ (Figure 5B). In peripheral blood, ~69.7% of WBCs were P-Dex-Alexa+, but virtually no signal was seen in red blood cells (data not shown).

**Renal distribution of P-Dex-Alexa**

To identify the mechanism responsible for renal retention of P-Dex, mice were injected with P-Dex-Alexa, and 7 days later, kidneys were isolated and analyzed by fluorescence microscopy and FACS. P-Dex-Alexa+ cells were most abundant in proximal tubules of the renal cortex (Figure 5C). Immunohistochemical staining indicated that a large proportion of retained prodrug was localized within cortical epithelial cells, which were identified based upon histology and E-cadherin positivity (Figure 5D). Flow cytometry indicated that 21.3% of the P-Dex-Alexa+ kidney cells were E-cadherin+ (data not shown). The identity of the remaining P-Dex-Alexa+ cells could not be determined definitively by flow cytometry, despite the use of an extensive panel of antibodies designed to identify various cells in the inflamed kidney. Less than 0.1% of the P-Dex-Alexa+ cells in kidney were F4/80+ macrophages, CD11c+ dendritic cells, CD4+ helper T cells or CD8a+ cytotoxic T cells (data not shown).

**Internalization, intracellular localization and activation of P-Dex**

Proximal tubule epithelial cells contribute to nephritis by secreting chemokines and inflammatory cytokines in response to albumin and immune complexes [14–17]. Because these cells represent the largest defined population of P-Dex-Alexa+ cells, the internalization kinetics of P-Dex-FITC was examined in HK-2 renal proximal tubule cells in vitro. HK-2 cells rapidly internalized P-Dex-FITC (Figure 5A). To test the hypothesis that renal inflammation enhances uptake of P-Dex, we examined prodrug uptake in HK-2 cells treated with LPS, which causes renal inflammation in vivo and induces the release of proinflammatory cytokines and mediators from renal cells in vitro [18, 19]. LPS did not alter prodrug internalization kinetics (Figure 6A).

To examine the fate of internalized P-Dex-FITC, we used immunohistochemistry and confocal microscopy. Internalized P-Dex-FITC co-localized with the LysoTracker® lysosome marker in HK-2 cells (Figure 6B), suggesting that P-Dex-FITC is internalized and processed by an endocytic pathway, that results in sequestration in a lysosomal compartment, where P-Dex would gradually undergo processing in the acidic environment, leading to the release of active drug [2–4].

To examine the impact of P-Dex on LPS-induced cytokine release in HK-2 cells, ELISA was used to evaluate secretion of the proinflammatory cytokine IL-6 into the supernatant. Although untreated HK-2 cells secrete low levels of IL-6, secretion can be stimulated 60-fold by LPS (Figure 6C; $P < 1 \times 10^{-5}$). Dex and P-Dex significantly attenuated the LPS-induced increase in IL-6 secretion (Figure 6C; $P < 5 \times 10^{-3}$), indicating that both treatments can inhibit secretion of inflammatory cytokines by activated proximal tubule epithelial cells.

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Discussion

Recent lupus drug development strategies have focused on targeting specific molecules and pathways that impact immunologic and proinflammatory processes. Although progress has been made, off-target toxicity due to the inability to manage in vivo drug distribution still poses a significant clinical challenge. Targeting inflammation with macromolecular prodrugs is a new nanomedicine-based therapeutic strategy. This approach is based on a mechanism involving extravasation of macromolecules through leaky vasculature and inflammatory cell-mediated sequestration (ELVIS) and has been validated in several inflammatory disease models [2, 4, 20].

Based upon these studies, we hypothesized that a dexamethasone prodrug could selectively target lupus-associated renal inflammation and become activated locally to ameliorate nephritis. Furthermore, we postulated that this prodrug would avoid the off-target toxicity associated with traditional GC therapy. To test this hypothesis, we treated (NZB×NZW)F1 mice with saline, PHPMA, Dex, or P-Dex beginning at 4 months of age, prior to the onset of albuminuria. Over the next 8 weeks, the majority of mice treated with saline or PHPMA developed albuminuria. As expected, Dex decreased the incidence of albuminuria by ~50%, and reduced both serum anti-dsDNA IgG levels and renal immune complexes [21]. Strikingly, P-Dex was more effective than Dex and completely prevented albuminuria. P-Dex did not affect serum anti-dsDNA IgG levels or renal immune complexes, but did reduce renal macrophage infiltration. These observations suggest that P-Dex may attenuate nephritis by reducing renal inflammation and act via different mechanisms than free Dex. Importantly, our results also demonstrate that P-Dex does not cause osteoporosis, a major systemic side effect associated with GC treatment.

Based upon the above data and the ELVIS mechanism, we hypothesized that P-Dex would extravasate and be retained at sites of renal inflammation. Optical imaging, immunohistochemistry and FACS analysis confirmed preferential accumulation and retention of P-Dex in inflamed kidneys of lupus-prone but not healthy control mice. Furthermore, these in vivo data validate that cell sequestration is the major mechanism for retention of P-Dex in the inflamed kidney, with proximal tubule epithelial cells being the primary cellular reservoir of sequestered P-Dex.

Our data, together with previously published work, support a model in which reduced glomerular capillary macromolecular permselectivity associated with renal inflammation enhances passage of P-Dex into the ultrafiltrate. This in turn leads to uptake and sequestration of significant quantities of the prodrug by the activated proximal tubule epithelium. The glomerulus is responsible for forming a nearly protein-free plasma ultrafiltrate. The essential components of the glomerular filtration barrier, the barrier between the blood and urinary space, are the fenestrated capillary endothelium, the surrounding basement membrane, and the filtration slit (spanned by the slit diaphragm) between adjacent foot processes of podocytes. In the normal kidney, small solutes readily pass through the filtration barrier, with passage increasingly restricted as molecular weights exceed 15 kDa; solutes of >50–60 kDa have very limited passage into the ultrafiltrate. Macromolecules having a net negative charge are further impeded from crossing the filtration barrier. Given that P-Dex is a neutral molecule of ~36 kDa, some P-Dex filtration is likely under normal conditions. Loss of integrity of the filtration barrier results in enhanced permeability (reduced permselectivity) of large molecules such as albumin, leading to albuminuria. Nephritis in (NZB×NZW)F1 mice is associated with vascular damage, alterations in the glomerular basement membrane and distortion of slit diaphragms [22–24], and this likely accounts for the enhanced filtration of P-Dex in these mice. The higher proportion of plasma P-Dex likely passing into the ultrafiltrate in (NZB×NZW)F1
mice compared to controls, results in increased P-Dex delivery to the apical aspect of the renal tubular epithelium. Proximal tubule epithelial cells reabsorb multiple substances from the tubular fluid, and previous reports indicate that polymer carriers can achieve renal targeting through uptake by these cells [25–29]. Enhanced prodrug delivery to and uptake by proximal tubule epithelial cells likely contributes to the efficacy of P-Dex.

Albumin and immune complexes activate proximal tubule epithelial cells and induce secretion of chemokines and cytokines such as IL-6 that promote renal inflammation and immune cell infiltration [14–17]. Our in vitro data indicate that P-Dex, similar to free Dex, inhibits LPS-induced IL-6 release from proximal tubule epithelial cells, suggesting that P-Dex may reduce renal inflammation by attenuating the pro-inflammatory response of proximal tubule epithelial cells. We postulated that inflammation would enhance endocytosis and P-Dex uptake in proximal tubule epithelial cells. Though not supported by our cell culture studies, this hypothesis was consistent with results from our in vivo studies, which indicate that kidney cells in the (NZB×NZW)F1 mice take up and retain larger quantities of P-Dex than those in NZW mice.

Although proximal tubule epithelial cells were identified as a major population of P-Dex-Alexa488+ cells in kidney, the identity of a substantial percentage of P-Dex-Alexa488+ cells in kidney remains to be determined. Furthermore, P-Dex was also found in liver, spleen and peripheral WBCs of (NZB×NZW)F1 mice, which is consistent with our findings in other disease models [2, 4, 30]. A clear understanding of how P-Dex uptake or retention in these cells or tissues impacts the ability of P-Dex to prevent nephritis and reduce off-target toxicities needs further investigation.

In summary, monthly administration of P-Dex provided superior prevention of lupus nephritis and reduced toxicity in (NZB×NZW)F1 mice, as compared to dose equivalent, daily administered Dex. We speculate that the nephrotropism and retention of P-Dex in (NZB×NZW)F1 mice is, at least partially, attributed to reduced glomerular capillary permselectivity and enhanced uptake by activated kidney cells including proximal tubule epithelial cells. The intracellular processing of P-Dex into free dexamethasone and sustained release of active drug at the site of inflammation provides a rational explanation for the superior, sustained anti-inflammatory effect of P-Dex in the local environment. These data provide a rationale for the future development of this macromolecular prodrug system as a potential preventive and/or therapeutic agent for lupus patients. Further clarification of the mechanisms underlying P-Dex action will be essential for its structural optimization and clinical translation.

Acknowledgments

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References


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Figure 1.
P-Dex prevents albuminuria and reduces glomerular damage in (NZBxNZW)F1 females. A, The chemical structure of P-Dex prodrug is shown. B, Albuminuria data for mice in saline (N=10), PHPMA (N=10), Dex (N=15), and P-Dex (N=15) treatment groups is illustrated at the pretreatment (PT) and 8-week time points. The incidence of albuminuria at the 8-week time point for each group is shown (in %) in upper right corner of each sub-section. C, Quantification of the abnormal glomeruli frequency in each treatment group is shown. D, Representative PAS stained histological sections from each treatment group are provided. Scale bars: 50 μm. (*, $P < 1 \times 10^{-3}$; **, $P < 5 \times 10^{-4}$).
Figure 2.
The effect of different treatments on serum anti-dsDNA IgG and renal immune complexes. 
A. Anti-dsDNA IgG levels were determined via ELISA at pretreatment, 4-week, and 8-week time points. 
B. Representative sections of kidney from each treatment group are shown. 
Sections were stained for renal deposition of anti-dsDNA IgG via immunohistochemistry. 
Scale bars: 50 μm. 
C. Quantification of immune complex staining is illustrated. (*, \( P < 0.02 \); **, \( P < 0.001 \); ***, \( P < 0.0005 \))
Figure 3. Impact of treatments on renal macrophage infiltration in (NZB×NZW)F1 mice. A. Representative confocal images of immunohistochemical staining of kidney sections from each treatment group are shown. Sections were stained with an anti-Iba-1 antibody (red) and DAPI (blue). Negative control (no Iba-1 antibody) and merged images are shown. Scale bars: 25 μm. B. Quantification of Iba-1 staining is illustrated. (*, P < 0.05).
Figure 4.
Analysis of side effects associated with treatments in (NZB×NZW)F1 mice. A, Mean arterial pressure was measured at pretreatment, 4-week, and 8-week time points via tail-cuff method. The horizontal line represents the MAP (109±2) for a group of non-autoimmune NZW females (N=12) between 4 and 6 months of age. After 8 weeks of treatments, femurs were collected at necropsy for the endpoint analysis of bone quality. Mean bone mineral density (B) and trabecular thickness (C) measurements in each treatment group are shown. D, Total serum IgG levels was determined via ELISA at pretreatment and 8-week time points. (*, P < 0.05; **, P < 0.001; ***. P < 0.0005)
Figure 5.
The nephrotropism and renal cell retention of P-Dex in (NZB×NZW)F1 mice. A, Representative optical images of organs (i.e. heart, Ht; liver, Lv; spleen, Sp; lung, Lu; and kidneys, Kd) isolated from (NZB×NZW)F1 (upper panels) and NZW mice (lower panels) were taken at 2 d (left panels) or 7 d (right panels) post i.v. injection of P-Dex-IRDye. B, Representative images from FACS analysis of cells isolated from organs of (NZB×NZW)F1 or NZW mice without (black lines) or with P-Dex-Alexa treatment (blue filled) at 7 d post-injection. C, Representative fluorescence microscopy images of kidney sections from (NZB×NZW)F1 mice without or with P-Dex-Alexa treatment at 7 d post-injection. D, Representative confocal images of immunohistochemical staining of kidney sections from (NZB×NZW)F1 mice without or with P-Dex-Alexa treatment at 7 d post-injection. Sections were stained with an anti-mouse E-cadherin antibody and DAPI. Antibody signal (red), P-Dex-Alexa signal (green), DAPI signal (blue) and a merged image are shown. Scale bars: C, 150 μm; D, 15 μm.
Figure 6.
The *in vitro* internalization, intracellular localization and activation of P-Dex-FITC in HK-2 cells. A, Quantification of the internalized P-Dex-FITC in untreated and LPS-stimulated (10 μg/mL) HK-2 cells over a 72 h time course. B, Representative confocal images show internalization and intracellular localization of P-Dex-FITC in LPS-stimulated (10 μg/mL) HK-2 cells. LysoTracker signal (red), P-Dex-FITC signal (green), DAPI signal (blue) and a merged image is shown. Scale bar: 15 μm. C, The impact of Dex and P-Dex on LPS-induced IL-6 secretion in HK-2 cells is shown. Results were tabulated from three separate experiments (*) , \( P < 5 \times 10^{-3} \); ** , \( P < 5 \times 10^{-5} \)