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Polymeric dexamethasone prodrugs attenuate lupus nephritis in MRL/lpr mice with reduced glucocorticoid toxicity

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Abstract

Due to their potent immunosuppressive and anti-inflammatory effects, glucocorticoids (GCs) are the most widely used medications in treating lupus nephritis (LN). Long-term use of GCs, however, is associated with numerous off-target adverse effects. To reduce GCs' adverse effects, we previously developed two polymeric dexamethasone prodrug nanomedicines: *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-based dexamethasone prodrug (P-Dex), and micelle-forming polyethylene glycol (PEG)-based dexamethasone prodrug (ZSJ-0228). Both P-Dex and ZSJ-0228 provided sustained amelioration of LN in lupus-prone NZB/W F1 mice with reduced GC-associated adverse effects. Here, we have extended our investigation to the MRL/lpr mouse model of LN. Compared to dose equivalent daily dexamethasone sodium phosphate (Dex) treatment, monthly P-Dex or ZSJ-0228 treatments were more effective in reducing proteinuria and extending the lifespan of MRL/lpr mice. Unlike the daily Dex treatment, ZSJ-0228 was not associated with measurable GC-associated adverse effects. In contrast, adrenal gland atrophy was observed in P-Dex treated mice.

Keywords

Lupus nephritis; Glucocorticoids; Dexamethasone; Polymeric prodrug; Toxicity

Background

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease associated with aberrant immune system regulation leading to a broad spectrum of clinical manifestations,

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Conflict of interest: Dong Wang and Zhenshan Jia are co-inventors of the patent (PCT/US16/61728), which covers the ZSJ-0228 prodrug technology. Dong Wang is a co-founder of Shannon Pharmaceuticals, which has licensed the ZSJ-0228 technology for commercial development and provides funding for Chemistry Manufacturing and Controls studies in Dong Wang's laboratory. Steven R. Goldring and Mary K. Crow are paid consultants for Shannon Pharmaceuticals.

including dermatitis, arthritis, pericarditis, neuropsychiatric disorders, and nephritis.[1-3] Due to the genetic and phenotypic heterogeneity of SLE, the diagnosis, treatment, and development of new therapies have been challenging.[4] Among the clinical manifestations of SLE, lupus nephritis (LN) is a major cause of morbidity and mortality and often requires intense and personalized care to prevent the progression of the nephritis.[5] LN is associated with the deposition of immune complexes in the glomeruli and renal tubules and the recruitment and activation of inflammatory cells, which lead to damage to the renal tissues and impaired renal function.[6] If left untreated, LN may progress to end-stage renal disease and eventually to renal failure.

Systemic glucocorticoids (GCs) are among the most widely used therapies for the treatment of LN related to their potent anti-inflammatory and immunosuppressive effects.[7] However, their use is associated with significant off-target toxicities that have hampered their long-term clinical use in treating LN.[8, 9] Recognizing the therapeutic efficacy of GCs and the limitations related to their off-target toxicities, we proposed to address this challenge by changing the biodistribution profile of GCs through polymeric prodrug nanomedicine design.

In an initial effort, we prepared an *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-based macromolecular prodrug of dexamethasone (P-Dex, Scheme 1A).[10] When tested in lupus-prone NZB/W F1 mice, P-Dex passively targeted the inflamed kidneys and exerted potent and sustained anti-inflammatory efficacy compared to dose equivalent free dexamethasone sodium phosphate (Dex) treatment.[11, 12] Importantly, no osteopenia was detected with the systemic administration of P-Dex. Other GC-associated adverse effects, including adrenal gland atrophy, however, were still present. To address these residual toxicities, we designed a polyethylene glycol (PEG)-based dexamethasone prodrug (ZSJ-0228, Scheme 1B).[13, 14] The amphiphilic molecules self-assemble into micelles in aqueous solutions. When assessed in NZB/W F1 mice, ZSJ-0228 showed potent efficacy in the treatment of nephritis, but importantly was not associated with GC adverse effects, including adrenal gland atrophy.[13, 14]

SLE is a complex autoimmune disease with heterogeneous clinical manifestations.[15-18] The heterogeneity is also observed in the spontaneous murine lupus models, where the disease may be driven by different pathogenic mechanisms.[19] For instance, MRL/lpr model seems to have higher levels of neutrophils and interstitial inflammation when compared to NZB/W F1 mice.[20] The pathogenic differences amongst distinct murine models of lupus can have important clinical implications and as a consequence, distinct models can respond differently to therapeutic interventions. Therefore, to further validate the efficacy and safety of P-Dex and ZSJ-0228, we have extended our initial studies in the NZB/W F1 mice to the MRL/lpr mouse model of lupus. The MRL/lpr mouse model recapitulates many of the clinical manifestations and immune dysregulation observed in human SLE, including the presence of skin lesions, which are not manifest in the NZB/W F1 mice.[20, 21]

Methods

Synthesis of polymeric dexamethasone prodrugs

P-Dex was synthesized via reversible addition-fragmentation chain transfer (RAFT) copolymerization of *N*-methacryloyl-glycyl-glycyl-hydrazyl-dexamethasone (MA-Dex) and HPMA using 2,2'-azobisisobutyronitrile as the initiator and *S*,*S*'-bis(α,α'-dimethyl-α''-acetic acid)-trithiocarbonate as the RAFT agent as reported previously.[10] The resulting P-Dex was then purified using a Sephadex LH-20 column (GE Healthcare, Chicago, IL) and dialyzed against water prior to lyophilization. The FPLC analysis showed that the weight average molecular weight (M_w) of P-Dex is 34 kDa with a dispersity ($Đ$) of 1.34. A previous *in vitro* release study has shown that Dex was gradually released from the P-Dex according to a zero-order kinetics at pH 5.0 with a releasing rate of ~ 1 wt%/day. At neutral pH, nearly no Dex release was detected.[10]

ZSJ-0228 was prepared and characterized as reported previously.[13, 14] As the first step, two dexamethasone molecules were conjugated to a glycine/glutamic acid/glycine linker system via hydrazone bonds to produce a dexamethasone dimer. The primary amine (from glutamic acid) of the dimer was then conjugated with the carboxylate terminus of mPEG 1900 to produce ZSJ-0228. In aqueous solutions, the amphiphilic ZSJ-0228 self-assembles into micelles.[13] The molecular weight of ZSJ-0228 is around 2979 Da. Dynamic light scattering measurements showed that the ZSJ-0228 can form micelles with an average size of 33 nm. The *in vitro* ZSJ-0228 release experiments showed a zero-order Dex release kinetics with a release rate of ~1.32 wt%/day at pH 4.5 and ~0.96 wt%/day at pH 5.0). No Dex release was detected at pH 6.5 and pH 7.4.[13]

Animal experiments

All of the experimental animals were kept under controlled temperature, humidity, and light conditions with food and water *ad libitum* in the University of Nebraska Medical Center (UNMC) animal facility, certified by the American Association for Accreditation of Laboratory Animal Care. All animal experiments were reviewed and approved by UNMC Institutional Animal Care and Use Committee and were performed following the Guide for the Care and Use of Laboratory Animals – 8th edition.

A total of 52 female MRL/lpr mice and 6 female MRL/MpJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). At 14-weeks, all of the MRL/lpr mice were randomized into four groups (13 mice/group). Three groups were given i.v. P-Dex (28 mg/kg dexamethasone dose equivalent), ZSJ-0228 (28 mg/kg dexamethasone dose equivalent), or saline every 4 weeks (on Day 0 and Day 28), respectively. The fourth group received daily intraperitoneal injection of dexamethasone sodium phosphate (Dex, 1 mg/kg/day dexamethasone equivalent). The total dose of P-Dex, ZSJ-0228, and Dex was equivalent in terms of dexamethasone. All treatments lasted for 8 weeks. Age-matched MRL/MpJ mice (6 mice) were used as healthy controls. During the entire course of the experiment, the proteinuria levels of the mice were tested weekly. Sera were collected every 4 weeks. After 8-week treatment, all of the surviving mice were euthanized, with major tissues/organs isolated and processed. It is important to note that the MRL/lpr mice have a

shorter life span than NZB/W F1 mice which we have used previously. Since the treatment study was initiated after LN onset, a longer than 8-week study duration would have resulted in an insufficient sample size in the Saline control group, compromising the inter-group comparison.

Proteinuria measurement

At pre-selected time points, individual urine samples were collected and applied to Siemens Albustix strips (Erlangen, Germany) for proteinuria measurement. A “negative” reading indicates the proteinuria levels were < 10 mg/dL. A reading of 1+, 2+, 3+ or 4+ indicate a proteinuria level of 10-30 mg/dL, 100-300 mg/dL, 300-2000 mg/dL, or > 2000 mg/dL, respectively.

Basal blood glucose test

Basal blood glucose levels were monitored every four weeks. After 8 hours of fasting, the blood glucose level was measured using a LifeScan OneTouch Ultra2 glucometer (LifeScan Inc., Milpitas, CA).

Blood collection from maxillary veins

Blood samples were collected from the maxillary vein.[22] Every 4 weeks, ~250 μL of blood was collected from the maxillary vein and then processed for analysis. A mouse bleeding lancet (MEDIpoint Inc., Mineola, NY) was used to pierce the skin in the maxillary area, and a red-cap serum tube was used to collect the blood. Afterward, the puncture site was gently pressed with sterile gauze to stop the bleeding.

Kidney histological analysis

After collection and processing, kidneys were sectioned into 3-micron slices. Slides were stained using periodic acid-Schiff (PAS). The histological analysis included assessment of inflammation, proliferation, crescent formation, and necrosis.[23] A total of 30 glomeruli per mouse were scored. For each glomerulus, a score of 0 – 4 was assigned, where 0 = normal, 1 = mild (with mesangial hypercellularity), 2 = moderate (with endocapillary hypercellularity or wire-loop lesion), 3 = severe (with crescent formation), and 4 = necrosis. Scores were summed and averaged to yield the glomerulonephritis score for an individual mouse.

Assessment of skin lesions

To assess the skin lesions, a score of 0 – 4 was assigned for each mouse. The grading system was adapted from a method published previously.[24] A score of 0 = no visible skin changes; 1 = hair loss with redness on snout and/or ears; 2 = hair loss with redness and a few scattered lesions on one or more of the following sites (snout, ears, and dorsal skin); 3 = redness, hair loss, and scabbing with a small area of involvement; 4 = ulcerations with an extensive area of involvement.

Serum immunoglobulin analysis

Serum anti-nuclear antibodies (ANA) and anti-dsDNA antibodies were detected by enzyme-linked immunosorbent assay (ELISA). Serum ANA levels were measured using mouse

anti-nuclear antigen ELISA kits (Alpha Diagnostic, Catalog# 5210). Serum anti-dsDNA antibodies were analyzed using mouse anti-dsDNA ELISA kits (Alpha Diagnostic, Catalog# 5110). The ELISA assays were performed following the manufacturer's instructions.

Immunohistochemistry and immunofluorescence staining

The renal immune complex deposition was evaluated by immunohistochemistry (IHC). For IHC examination, deparaffinized kidney sections were steamed for 10 minutes in 10 mM sodium citrate buffer using a steamer. Sections were then brought to room temperature and rinsed in a washing buffer (PBS, 0.5% Tween 20). After antigen retrieval, samples were blocked with 2.5% normal goat serum and then stained with ImmPRESS anti-mouse IgG (Vector MP-7802). The staining was visualized using a DAB working solution (Vector MP-7802). The staining levels (arbitrary gray units) were quantified using ImageJ software (Version 1.52, NIH).

Renal macrophage infiltration was assessed by immunofluorescent staining (IF). Following deparaffinization and antigen retrieval, the sections were blocked and incubated with rat anti-mouse F4/80 antibody eFluor 570 (eBioscience, Inc.). Thirty glomeruli per slide were investigated to count positively stained macrophages using an Olympus BX51 Fluorescence Microscope (Tokyo, Japan).

Bone quality analysis

To analyze the effects of the different treatments on bone quality and structure, the hind legs of mice were isolated and fixed in formalin at the time of euthanasia. The bones were then scanned using a micro-CT (Skyscan 1172, Bruker, Kontich, Belgium). The parameters were 55 kV, 181 μA, 0.5 mm aluminum filter, 9 μm resolution, 4 frames averaging, 0.4 rotation step, 180° scanning. To calculate the bone mineral density (BMD), two standard hydroxyapatite rods were also scanned to serve as standards. The raw images were reconstructed using NRecon software (version 1.7.4.6, Bruker microCT). All reconstructed images were registered and realigned before analysis using DataViewer software (version 1.5.6.2, Bruker microCT). The femoral bone was then evaluated using CTAn software (version 1.18.8.0, Bruker microCT). Bone mineral density (BMD), bone volume fraction (BV/TV), and trabecular thickness (Tr.Th) were calculated.

Statistical analysis

Statistical analyses of the data were performed using the Mann-Whitney U test. Percentage survival data were analyzed using the log-rank (Mantel-Cox) test. P values equal to or less than 0.05 were considered statistically significant. GraphPad Prism 7 software (version 7.00) was employed to perform all of the statistical analyses. Data were presented as mean ± SEM.

Results

Both P-Dex and ZSJ-0228 attenuated proteinuria and improved the survival rate of MRL/lpr mice

As shown in Figure 1A, by the end of the study, 9 out of 13 mice (69.2%) in the Saline group had developed nephritis, characterized by a proteinuria reading ≥ 2. By contrast, 3 out of 13 mice (38.5%) treated with P-Dex developed nephritis; 2 out of 13 mice (15.4%) treated with ZSJ-0228 developed nephritis; 6 out of 13 mice (46.2%) treated with Dex developed nephritis. The average proteinuria levels of the P-Dex treated mice were lower than that of the Saline group with borderline statistical significance ($P = 0.0542$). The average proteinuria levels of the ZSJ-0228 group were significantly lower than that of the Dex ($P < 0.05$) and the Saline groups ($P < 0.001$). These data suggest that both P-Dex and ZSJ-0228 treatment reduced the proteinuria levels of MRL/lpr mice compared to the Dex and Saline treated mice, with ZSJ-0228 outperforming P-Dex.

The survival curves of different groups were generally consistent with the proteinuria findings. As shown in Figure 1B, 92% of the mice treated with P-Dex and 100% of the mice treated with ZSJ-0228 survived after an eight-week treatment. Approximately 85% of the mice treated with daily Dex survived over the entire treatment period. In the Saline group, ~61% of the mice survived to the end of the study. ZSJ-0228 treatment significantly increased the survival rate of the mice compared to the Saline group ($P < 0.05$), providing evidence that ZSJ-0228 extended the lifespan of the MRL/lpr mice. The survival curve of the P-Dex group was more favorable than the Saline group but did not reach statistical significance ($P = 0.075$). Mice in the Dex group also had an improved lifespan compared to the Saline group.

Renal histological findings in mice treated with P-Dex and ZSJ-0228

To further assess the efficacy of P-Dex and ZSJ-0228, we performed the histological analysis of renal sections. Histological analysis of pathologic features of glomerulonephritis included assessment of inflammation, proliferation, crescent formation, and necrosis. Representative PAS-stained kidney sections are presented in Figure 2A. As shown in Figure 2B, the average kidney histological score of the P-Dex group was significantly lower than the Saline group ($P < 0.05$), consistent with reduced renal pathology in the P-Dex treatment group. ZSJ-0228 treated mice also exhibited significantly ($P < 0.001$) lower average kidney histological scores compared to the Saline treated mice and were lower than the P-Dex treated mice, confirming its superior therapeutic efficacy. Daily Dex treatment also reduced renal pathology, with significantly lower average kidney histological scores compared to the Saline group ($P < 0.05$).

P-Dex or ZSJ-0228 did not reduce serum ANA or anti-dsDNA antibody levels

Autoantibodies, particularly anti-nuclear antibodies (ANA) and anti-dsDNA antibodies, play a role in the development of SLE and LN.[25, 26] The P-Dex, ZSJ-0228, and Dex groups had serum ANA levels comparable to the Saline group after 8-weeks of treatment (Figure 3A), consistent with an absence of an effect of these treatments on the serum ANA levels. As shown in Figure 3B, the serum anti-dsDNA antibody levels in the Dex group were

significantly lower than in the P-Dex group ($P < 0.05$) and also lower than the ZSJ-0228 ($P = 0.0609$) and Saline groups ($P = 0.0674$). Daily Dex treatment reduced the anti-dsDNA antibody levels, which is consistent with the decreased proteinuria readings and lower kidney histological scores in the Dex group. In contrast, there was no significant difference in serum anti-dsDNA antibody levels among the P-Dex, ZSJ-0228, and Saline groups. Collectively, these data suggest that the therapeutic efficacy of P-Dex and ZSJ-0228 in treating LN was not dependent on an effect on serum autoantibodies.

Neither P-Dex nor ZSJ-0228 inhibited renal immune complex deposition

We next evaluated the effect of the treatments on renal immune complex deposition (Figure 4A).[27, 28] As shown in Figure 4B, the renal immune complex levels in the P-Dex and ZSJ-0228 groups were not significantly different from the Saline group, suggesting their beneficial effects on nephritis were independent of renal immune complex deposition. By contrast, the immune complex staining level in the Dex group was significantly lower than that of the P-Dex ($P < 0.05$) and the Saline groups ($P < 0.05$).

The impact of P-Dex and ZSJ-0228 treatments on renal macrophage infiltration

LN is characteristically associated with increased glomerular macrophage infiltration, which likely contributes to impaired renal function.[29] Figure 5A demonstrates the pattern of macrophage infiltration (identified by positive immunofluorescent F4/80 staining) in representative glomeruli. Quantitation of the number of F4/80 positive cells in representative glomeruli revealed that macrophage infiltration was significantly lower in the P-Dex ($P < 0.05$), ZSJ-0228 ($P < 0.01$), and Dex ($P < 0.01$) treated groups when compared to the Saline group (Figure 5B), consistent with a beneficial effect of the treatments on renal inflammation.

Effects of P-Dex or ZSJ-0228 on extra-renal lupus symptoms

In addition to nephritis, female MRL/lpr mice also develop multiple other lupus-related pathologies, including skin lesions, lymphadenopathy, and splenomegaly.[30, 31] To determine whether the treatment with P-Dex or ZSJ-0228 had an effect on ameliorating skin lesions, we analyzed the pattern and distribution of skin lesions using a semi-quantitative scoring system. As shown in Figure 6A, the skin lesion scores in the daily Dex, P-Dex, or ZSJ-0228 groups were similar to the score in Saline treated mice, suggesting that the treatments had limited impacts on MRL/lpr associated skin lesions.

The size of the abnormal lymph nodes and spleens were similar to the Healthy controls but were significantly smaller in MRL/lpr mice treated with the daily Dex when compared to the Saline group (Figure 6B, C), consistent with a favorable therapeutic effect on lupus-related lymphadenopathy and splenomegaly. By contrast, neither P-Dex nor ZSJ-0228 treatment had an effect on lymph node or splenic weights (Figure 6B, C).

MRL/lpr mice with established lupus pathology have elevated serum total IgG levels.[32] As shown in Figure 6D, we did not observe any effect of P-Dex or ZSJ-0228 treatment on total IgG serum levels. In contrast, the average total serum IgG levels in the Dex treated

group was similar to the healthy controls and significantly lower than the P-Dex ($P < 0.01$), ZSJ-0228 ($P < 0.001$), or Saline groups ($P < 0.05$).

Assessments of GC-associated adverse effects

Chronic use of GCs is accompanied by hypothalamic-pituitary-adrenal axis suppression and adrenal atrophy, which is associated with significant morbidity and even death.[33, 34] To explore the potential adverse effects of P-Dex and ZSJ-0228 on this axis, we measured adrenal gland weight for each mouse at necropsy. As shown in Figure 7A, the average adrenal gland weight in the Dex group was significantly lower than the Saline group ($P < 0.01$) and the Healthy control ($P < 0.01$), consistent with adrenal suppression related to daily Dex treatment. The average adrenal gland weight of the P-Dex group was significantly lower than the Healthy group ($P < 0.05$), indicating P-Dex treatment also induced adrenal suppression. In contrast, ZSJ-0228 treatment did not decrease adrenal gland weight compared to the Saline group and the Healthy controls.

Blood glucose levels were measured to explore the impact of the different treatments on glucose metabolism. Consistent with our previous study,[35] the daily Dex group showed significantly lower blood glucose levels after 8-week treatment when compared to the Saline ($P < 0.001$) and the Healthy control groups ($P < 0.001$, Figure 7B). There was no significant difference in mean blood glucose levels in the P-Dex, ZSJ-0228, Saline, and the Healthy control groups, suggesting neither P-Dex nor ZSJ-0228 monthly treatment affected glucose metabolism.

GC therapy is associated with systemic immunosuppression, with reduced peripheral white blood cell (WBC) counts. Analysis of the impact of different treatments on WBC counts revealed no significant difference in WBC counts in the P-Dex, ZSJ-0228, Saline, and the Healthy control groups (Figure 7C). However, the average WBC counts in the daily Dex group were significantly lower than in all other groups, except the Saline group ($P = 0.0864$). These results suggest that, unlike the daily Dex treatment, the ZSJ-0228 and P-Dex treatments did not decrease peripheral WBC counts.

The development of osteoporosis is another adverse effect associated with chronic GC administration. To explore the impact of P-Dex and ZSJ-0228 treatment on MRL/lpr mice bone structure and quality, micro-CT scans were performed on the femoral bones from mice in the different treatment groups. As shown in Figure 7D, no significant difference in bone mineral density (BMD) was observed among the P-Dex, ZSJ-0228, Saline, and Healthy groups. Mice treated with daily Dex had a significantly decreased BMD compared with the Saline ($P < 0.05$) and Healthy groups ($P < 0.05$). Similar trends were also observed in the trabecular bone volume fraction (BV/TV, Figure 7E). As shown in Figure 7F, trabecular thickness (Tr.Th) in the daily Dex group was significantly lower than all the other groups. Thus, unlike the daily Dex treatment, monthly P-Dex or ZSJ-0228 did not negatively affect the bone quality or structure of the MRL/lpr mice in the present study.

Discussion

GCs remain the most widely used medication for the treatment of LN. According to recent guidelines published by the American College of Rheumatology (ACR), high dose pulse GCs followed by daily GC plus an additional immunosuppressive drug is still the standard recommended treatment for LN,[36] and there is no recommended alternative for GCs. Chronic use of GCs, however, is associated with multiple significant adverse side effects through triggering non-target organ transactivation pathways, in part related to off-target biodistribution.[37] To address this challenge, we have employed a prodrug nanomedicine strategy that alters the pharmacokinetics and biodistribution (PK/BD) profiles of GCs. Previously, we successfully designed and tested two polymeric dexamethasone prodrug nanomedicines (P-Dex and ZSJ-0228) that showed significant therapeutic efficacy and decreased toxicity in treating LN in the NZB/W F1 mouse model of lupus. Recognizing the highly heterogeneous nature of SLE[38], we have extended these initial studies to assess the therapeutic efficacy and safety of monthly P-Dex or ZSJ-0228 in the MRL/lpr mouse model, which in addition to nephritis, exhibits multiple other lupus associated pathologies.

Both monthly P-Dex or ZSJ-0228 were effective in ameliorating LN after 8-weeks of treatment in MRL/lpr mice. ZSJ-0228 was more potent than P-Dex. Neither P-Dex nor ZSJ-0228 treatment affected serum ANA or anti-dsDNA antibody levels or renal deposition of immune complexes. However, both of the treatments reduced the renal infiltration of macrophages. These data suggest that P-Dex and ZSJ-0228 treatment may ameliorate LN primarily by reducing renal inflammation without affecting immune complex deposition. In addition to LN, female MRL/lpr mice also develop additional pathologies, including skin lesions, lymphadenopathy, and splenomegaly. Neither P-Dex nor ZSJ-0228 treatment had an effect on these extra-renal lupus pathologies. By contrast, daily Dex treatment ameliorated lymphadenopathy and splenomegaly but did not affect skin lesions. We speculate that the unique anatomical/physiological features of the skin compartment may have resulted in limited access to the tested medications. This is supported by the demonstrated efficacy of topical GC formulations in managing certain forms of cutaneous lupus.[39]

Importantly, no GC-associated adverse effects (*e.g.,* adrenal gland atrophy, blood glucose reduction, WBC counts reduction, or bone loss) were observed in the MRL/lpr mice treated monthly with ZSJ-0228, which is consistent with the results presented in the previous NZB/W F1 mouse studies.[11, 12] P-Dex treatment was associated with adrenal gland atrophy, but no other GC-associated adverse effects were observed with this formulation. The different safety profiles of ZSJ-0228 and P-Dex may be explained by their different PK/BD patterns. Previously, we performed a head-to-head comparative PK/BD study of ZSJ-0228 and P-Dex in NZB/W F1 mice.[40] The PK/BD study revealed that mice treated with Iodine-125 labeled P-Dex demonstrated a significantly higher systemic and tissue exposure than mice treated with Iodine-125 labeled ZSJ-0228. Also, the free Dex released from P-Dex in all organs/tissues was higher than the free Dex released from ZSJ-0228. The presence of ZSJ-0228 in the spleen and adrenal glands was transient, while the low-level presence of P-Dex in the spleen and adrenal glands persisted for more than several weeks, which may potentially be attributed to its cell-mediated sequestration. We speculate that the PK profile of P-Dex in the spleen may be different in MRL/lpr mice since the P-Dex

treatment did not inhibit the development of splenomegaly in MRL/lpr mice as was found in the NZB/W F1 mice.

As expected, daily Dex treatment induced multiple GC-associated adverse effects, including adrenal gland atrophy, WBC count reduction, and osteopenia. Consistent with our observations in the NZB/W F1 mouse treatment study,[35] daily Dex also significantly decreased the blood glucose levels in MRL/lpr mice (Figure 7B). While this observation is opposite to what has been observed in humans, where GCs are known to induce hyperglycemia, it is clear that daily Dex treatment can interfere with glucose metabolism in the MRL/lpr mice. Overall, these adverse effects contribute significantly to morbidity in animal models and human patients with lupus.[41]

In summary, monthly P-Dex and ZSJ-0228 treatments provided potent amelioration of nephritis in lupus-prone female MRL/lpr mice. Comparing dose equivalent daily Dex treatment, P-Dex treatment mitigated multiple GC-associated adverse effects but was still associated with adrenal gland atrophy. ZSJ-0228 treatment resulted in a further improvement in the safety profile with no adrenal gland atrophy detected. As a limitation of this study, we recognize that the full impact of P-Dex and ZSJ-0228 on additional tissues and organs such as the liver and body fat need to be assessed. A comprehensive long-term safety evaluation is necessary to fully established the polymeric dexamethasone prodrugs' safety in the MRL/lpr mouse model. Given P-Dex and ZSJ-0228's unique PK/BD profiles,[40] additional in-depth mechanistic studies are necessary to better understand the sustained therapeutic benefits of these monthly polymeric prodrug treatments. Nevertheless, the results obtained in the MRL/lpr mouse model confirm the potential utility of polymeric prodrugs in the treatment of LN. Together with our previous findings in the NZB/W F1 mouse model, the present results in the MRL/lpr mouse model provide further support for the clinical development and translation of polymeric prodrug nanomedicines as potential novel therapies for LN in individuals with SLE.

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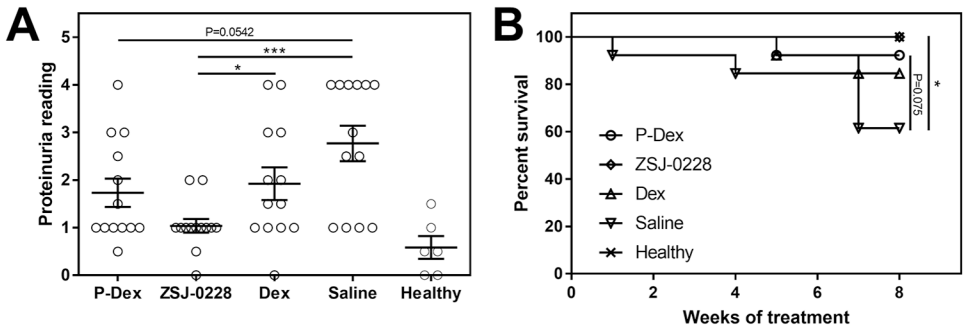


Figure 1.

Therapeutic effects of different treatments in ameliorating proteinuria and improving the survival rate of MRL/lpr mice. **A.** Proteinuria levels for the different treatment groups at the end of the study. For mice that died during the course of treatment due to severe lupus nephritis, their proteinuria readings were labeled as 4. Data were presented as mean ± SEM. Mann-Whitney U test, * $P < 0.05$, *** $P < 0.001$. **B.** Survival curves of different treatment groups. Log-rank (Mantel-Cox) test, * $P < 0.05$.

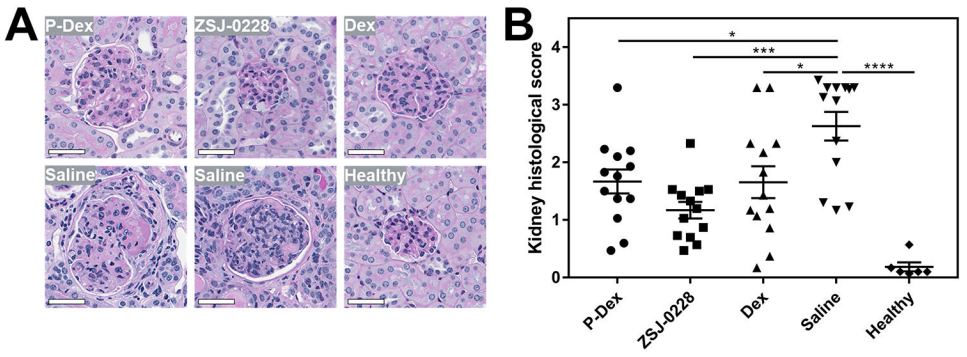


Figure 2.

P-Dex and ZSJ-0228 attenuated the renal pathological changes. **A.** Representative kidney sections for each group. Scale bar = 50 μm. **B.** Semi-quantitative kidney histological scores in the different treatment groups. Data are presented as mean ± SEM. Mann-Whitney U test, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

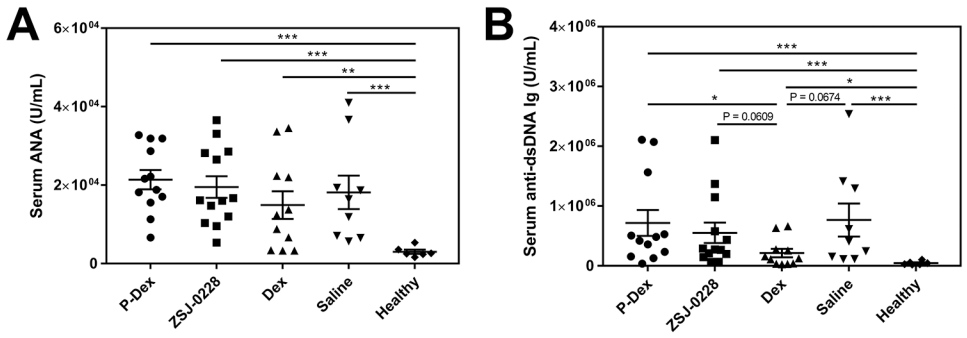


Figure 3.

Treatment effects on serum ANA and anti-dsDNA Ig levels. **A.** Serum ANA levels in each group at the end of the study. **B.** Serum anti-dsDNA antibodies levels in each group at the end of the study. Only mice that survived were analyzed for serum antibody levels. Data are presented as mean ± SEM. Mann-Whitney U test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

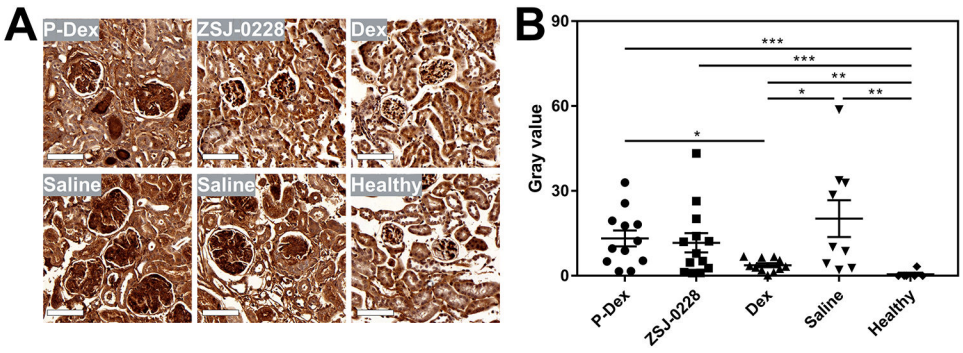


Figure 4.

Effect of the different treatments on renal immune complex deposition. **A.** Representative images of immune complex immunohistochemical staining from each group. Scale bar = 100 μm. **B.** Quantification of renal immune complex deposition. Data are shown as mean ± SEM. Mann-Whitney U test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

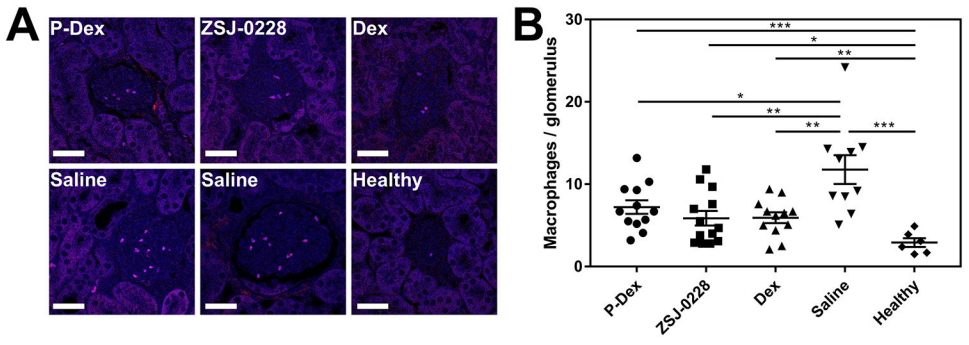


Figure 5.

Effect of the different treatments on glomerular macrophage infiltration. **A.** A glomerulus is present in the central area of each image (Scale bar = 50 μm). Glomerular macrophages are identified by positive F4/80 immunofluorescent-staining. **B.** The quantitative analysis of macrophage infiltration in each group. Mann-Whitney U test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are displayed as mean ± SEM.

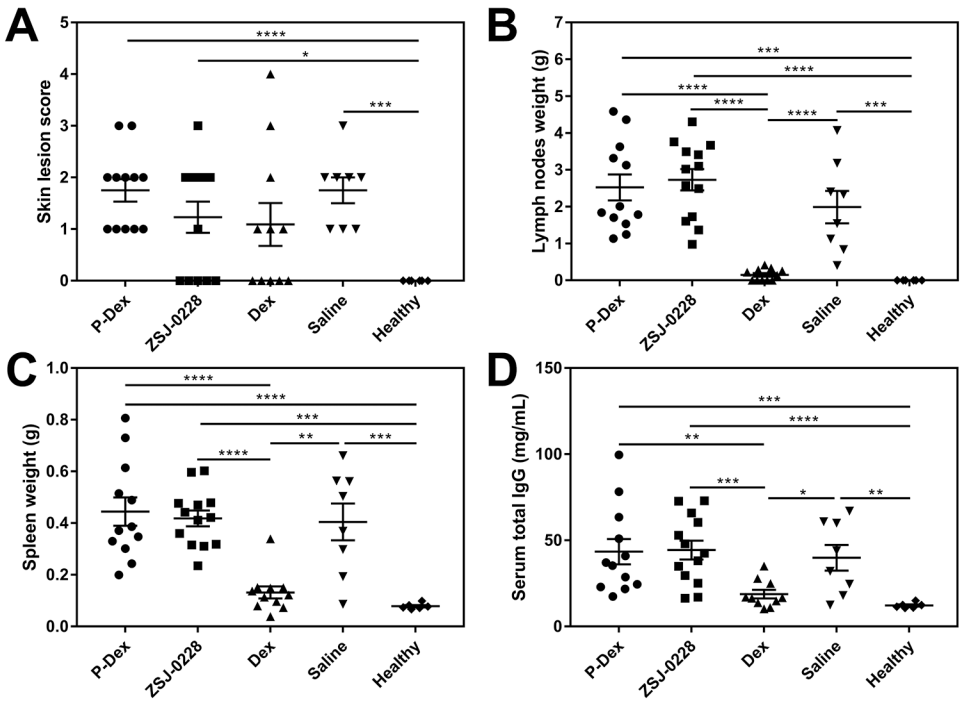


Figure 6.

The therapeutic effect of different treatments on extra-renal lupus-associated pathologies. **A.** Semi-quantitative analysis of skin lesions. **B.** Lymph node weight assessment. **C.** Splenic weight analyses. **D.** Serum total IgG levels assessment. Data are presented as mean ± SEM. Mann-Whitney U test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

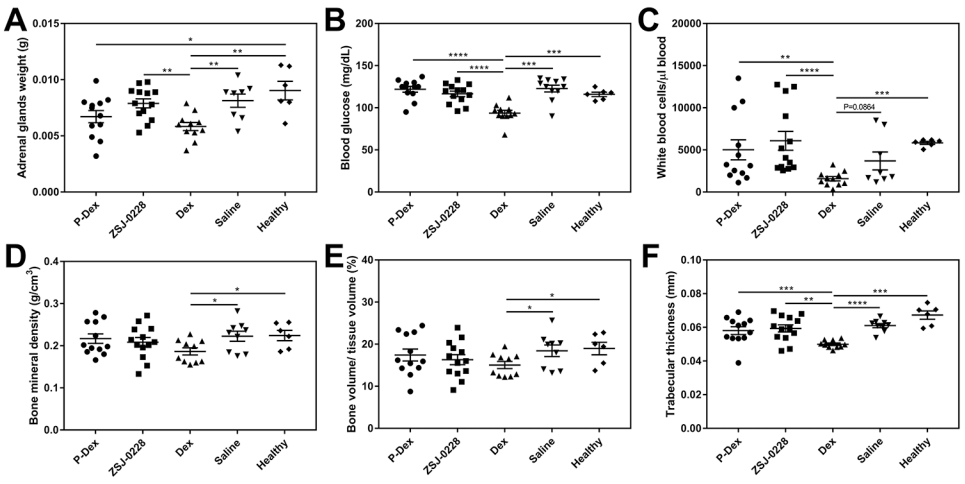
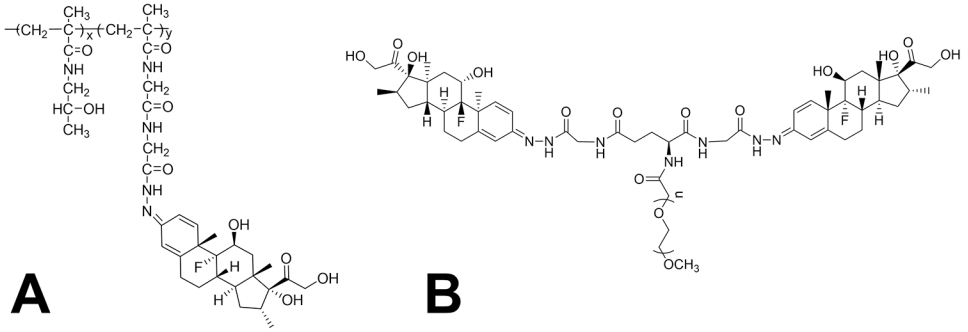


Figure 7.

Assessment of GC-associated toxicities induced by different treatments. **A.** Adrenal gland weight in the different treatment groups. **B.** Basal blood glucose levels of each group at the end of the study. **C.** Peripheral white blood cell counts. **D.** Bone mineral density (BMD). **E.** Bone volume to tissue volume ratio (BV/TV). **F.** Trabecular thickness (Tr.Th). Data are presented as mean ± SEM. Mann-Whitney U test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.



Scheme 1.

The design of dexamethasone prodrug nanomedicines. **A.** The structure of P-Dex, which is a dexamethasone prodrug based on HPMA copolymer. **B.** ZSJ-0228 assembles into micelles in aqueous solutions due to its unique amphiphilic structural design.