Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Mol Pharm. Author manuscript; available in PMC 2022 November 01.

Published in final edited form as:

Mol Pharm. 2021 November 01; 18(11): 4188–4197. doi:10.1021/acs.molpharmaceut.1c00567.

Preclinical Dose-escalation Study of ZSJ-0228, a Polymeric Dexamethasone Prodrug, in the Treatment of Murine Lupus Nephritis

Zhifeng Zhao[1], **Xiaoke Xu**[1], **Haochen Jiang**[1], **Kirk W. Foster**[2], **Zhenshan Jia**[1], **Xin Wei**[1], **Ningrong Chen**[1], **Steven R. Goldring**[3], **Mary K. Crow**[3], **Dong Wang**[1,*]

[1]Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE 68198-6125

[2]Department of Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, Omaha, NE, 68198-5900

[3]Hospital for Special Surgery, New York, NY, 10021

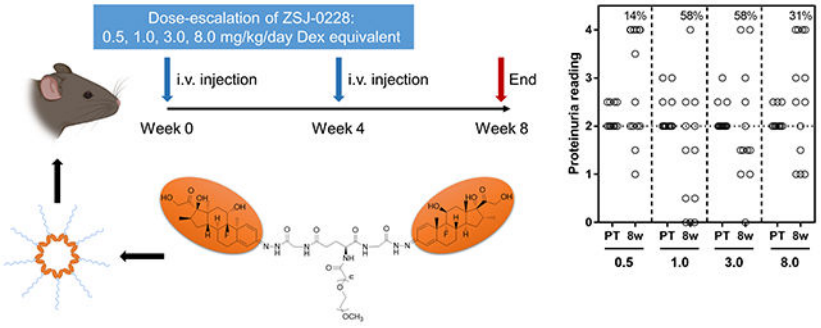
Abstract

Glucocorticoids (GCs) are widely used in the clinical management of lupus nephritis (LN). Their long-term use, however, is associated with the risk of significant systemic side effects. We have developed a polyethylene glycol (PEG)-based dexamethasone (Dex) prodrug (*i.e.,* ZSJ-0228) and in a previous study demonstrated its' potent therapeutic efficacy in mice with established LN, while avoiding systemic GC-associated toxicity. In the present study, we have employed a dose-escalation design to establish the optimal dose response relationships for ZSJ-0228 in treating LN and further investigated the safety of ZSJ-0228 in lupus-prone NZB/W F1 mice with established nephritis. ZSJ-0228 was intravenously (i.v.) administered monthly at four levels: 0.5 (L1), 1.0 (L2), 3.0 (L3), and 8.0 (L4) mg/kg/day Dex equivalent. For controls, mice were treated with i.v. saline every 4 weeks. In addition, a group of mice received intraperitoneal injections (i.p.) of Dex every day, or i.v. injections of Dex every four weeks. Treatment of mice with LN with ZSJ-0228 dosed at L1 resulted in resolution of proteinuria in 14% of the mice. Mice treated with ZSJ-0228 dosed at L2 and L3 levels resulted in resolution of proteinuria in ~60% of the mice in both groups. Treatment with ZSJ-0228 dosed at L4 resulted in resolution of proteinuria in 30% of the mice. The reduction and/or resolution of the proteinuria, improvement in renal histological scores and survival data indicate that the most effective dose range for ZSJ-0228 in treating LN in NZB/W F1 mice is between 1.0 and 3.0 mg/kg/day Dex equivalent. Typical GC-associated side effects (*e.g.,* osteopenia, adrenal glands atrophy, etc.) were not observed in any of the ZSJ-0228 treatment groups, confirming its excellent safety profile.

Graphical Abstract

*Correspondence should be addressed to Dong Wang, 986125 Nebraska Medical Center, PDD 3020, Omaha, NE 68198-6125, USA. Phone: +1 402 559 1995. Fax: +1 402 559 5643. dwang@unmc.edu.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript



Keywords

Dexamethasone; Polymeric prodrug; Lupus nephritis; Dose-escalation; Toxicity

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the loss of tolerance to self-antigens, formation of autoantibodies, and generalized immune dysregulation leading to the development of inflammation and tissue damage to multiple organs.[1] The clinical manifestations of SLE are heterogeneous, involving one or more organs, including the kidneys, joints, skin, and nervous system.[2] Lupus nephritis (LN) affects up to 60% of SLE patients within ten years of diagnosis and is an important contributor to mortality of the disease.[3–5]

Systemic (oral or parenteral) glucocorticoids (GCs) possess potent anti-inflammatory and immunomodulatory properties and are among the first-line medications used to treat LN.[6–8] However, chronic use of GCs is associated with serious side effects involving the musculoskeletal, gastrointestinal, cardiovascular, endocrine, neuropsychiatric, dermatologic, ocular, and immune systems.[9, 10] Moreover, because children with LN tend to have more active disease over time, they are at risk of exposure to more intense immunosuppressive treatments and are therefore at higher risk for the development of more serious adverse effects of GC toxicity.[11, 12]

In the 70-year history of GCs clinical use, there has been minimal progress in reducing the adverse effects of GCs. The biological activities of GCs are mediated through both transrepression and transactivation.[13] Evidence suggests that transrepression is the main pathway responsible for the anti-inflammatory effects of GCs, while transactivation mediates the GC-associated adverse effects.[14] Selective GC receptor modulators that preferentially stimulate the transrepression pathway vs. the transactivation pathway have been developed.[15–17] However, these drug candidates failed to show strict selectivity and continued to demonstrate GC-associated adverse effects. In the 1980s, several studies reported that the use of GCs at lower doses helped to reduce GC-associated adverse effects. However, this came at the expense of compromised therapeutic efficacy.[18, 19] To address this challenge, we employed a prodrug nanomedicine strategy to modify the pharmacokinetic/biodistribution (PK/BD) profile of GCs. Specifically, we designed

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and synthesized a polyethylene glycol-based dexamethasone (Dex) prodrug (ZSJ-0228) micelle formulation.[20, 21] When tested in lupus-prone female NZB/W F1 mice, ZSJ-0228 altered the pharmacology of Dex by focusing its biodistribution to the kidney, providing marked improvement in LN pathology and renal function as assessed by the levels of proteinuria. The therapeutic efficacy of ZSJ-0228 was superior to dose equivalent free Dex in ameliorating nephritis and was without measurable GC-associated adverse effects.[20]

In this manuscript, we report a preclinical dose-escalation study to further investigate the therapeutic potential of ZSJ-0228 in managing murine LN. The primary objective of this study was to identify the optimal dose range of ZSJ-0228. We also sought to explore the safety profile of ZSJ-0228 at higher dosing levels since the GC-associated adverse effects are known to be dose-dependent.[22, 23]

Experimental Methods

Ethics statement

All the experimental animals were kept under controlled lighting, temperature, and humidity conditions in the University of Nebraska Medical Center (UNMC) animal facility, certified by the American Association for Accreditation of Laboratory Animal Care (AAALAC). All procedures involving live animals were performed in compliance with protocol 16-035 approved by UNMC Institutional Animal Care and Use Committee (IACUC) and in compliance with the Guide for the Care and Use of Laboratory Animals – 8th edition.

Animal experiments

Lupus-prone female NZB/W F1 mice (20-week-old, 88 in total, Jackson Laboratory) were used in the study. The mice were monitored weekly for proteinuria and body weight. Only mice with established nephritis (around 30-week-old) were enrolled in the study and randomized into seven groups. Four groups were given i.v. injections of increasing doses of ZSJ-0228 every four weeks. The structural information (*e.g.*, molecular weight, Dex content, critical micelle concentration or CMC, micelle diameter) of ZSJ-0228 has been reported previously.[20] The dosing levels of ZSJ-0228 were set at 0.5 (L1, n=14), 1.0 (L2, n=12), 3.0 (L3, n=12), and 8.0 (L4, n=13) mg/kg/day Dex equivalent. As positive controls, a group of mice received i.p. injections of dexamethasone sodium phosphate every day (1.0 mg/kg/day Dex equivalent, daily Dex, n=12), and another group received i.v. injections of dexamethasone sodium phosphate every four weeks (1.0 mg/kg/day Dex equivalent, monthly Dex, n=12). The last group (n=13) was administered i.v. injections of saline every four weeks for negative controls. All treatments lasted for eight weeks. Young NZB/W F1 mice (16 weeks old) were used to demonstrate the histological structure, renal immune complex deposition, and renal macrophage infiltration of healthy kidneys. Serum was isolated from peripheral blood collected monthly via the submandibular vein. Basal blood glucose levels were tested using OneTouch Blood Glucose Meter (LifeScan, Malvern, PA). Mice with severe proteinuria (≥ 2000 mg/dL) or mice that displayed symptoms of distress (*i.e.*, edema, weight loss more than 20%, ungroomed appearance, reduced mobility) were euthanized immediately. At the end of the eight-week treatment, all surviving animals were sacrificed, with all major organs isolated and processed.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Proteinuria assessment

Proteinuria levels were measured by Siemens Albustix strips (Erlangen, Germany). The reading results correspond with the following proteinuria levels: negative (0 mg/dL), trace (10 mg/dL), 1+ (10-30 mg/dL), 2+ (100-300 mg/dL), 3+ (300-2000 mg/dL), 4+ (>2000 mg/dL). Nephritis was confirmed by two consecutive Albustix readings of 2 or above.

Histological analysis of kidney pathology

All of the collected kidneys were fixed by formalin, embedded in paraffin, sectioned at 3 microns, and stained with periodic acid-Schiff (PAS). Grading of the nephritis was performed using a semi-quantitative 0 – 4 scale, as reported previously.[24] In brief, a total of 50 glomeruli from each slide were analyzed, and abnormal glomeruli (*i.e.*, with hypercellularity, wire-loop lesions, crescent lesions) were counted. Scores of 0, 1, 2, 3, and 4 were assigned as follows: 0, 1-20, 21-50, 51-75, 76-100% of the glomeruli were abnormal, respectively.

Renal immune complex deposition and macrophage infiltration analysis

Renal immune complex deposition was evaluated by immunohistochemistry (IHC), as described previously.[21] The slides were visualized using an Olympus BX51 light microscope (Tokyo, Japan). Thirty glomeruli per slide were analyzed for the staining levels (arbitrary gray units) using ImageJ software (Version 1.52, NIH).

Renal macrophage infiltration levels were assessed by immunofluorescent staining (IF).[21] In brief, kidney slides were blocked and then incubated with rat anti-mouse F4/80 antibody eFluor 570 (eBioscience). Thirty glomeruli per slide were investigated to count positively stained macrophages.

Serum immunoglobulin analysis

Serum immunoglobulin concentrations were detected by enzyme-linked immunosorbent assay (ELISA).[25] Serum anti-dsDNA IgG levels were measured using the mouse anti-dsDNA IgG ELISA kit (Alpha Diagnostic, San Antonio, TX). Serum total IgG levels were analyzed using the mouse IgG ELISA kit (Innovation Research, Novi, MI). The analyses were performed according to the manufacturers' instructions.

Bone quality analysis

The bone quality was assessed using a Skyscan 1172 micro-CT system (Bruker, Kontich, Belgium). The micro-CT scanning parameters were set as follow: current: 181 μA; voltage: 55 kV; resolution: 9 μm; filter: 0.5 mm aluminum. Reconstructions were performed using NRecon software (version 1.7.4.6, Bruker microCT) and DataViewer software (version 1.5.6.2, Bruker microCT). A constant region of interest, from 20 slices to 100 slices proximal to the growth plate of the trabecular bone at the distal femur, was chosen for bone quality analysis. Bone mineral density (BMD), bone volume/tissue volume (BV/TV), trabecular thickness (Tr.Th), and trabecular number (Tr.N) were determined with CTAn software (version 1.18.8.0, Bruker microCT).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Statistical analysis

The one-way ANOVA with post hoc Tukey's test, Wilcoxon signed-rank test, or Mann-Whitney U test were performed to calculate statistically significant difference where appropriate. Data for the percent survival were analyzed with the log-rank (Mantel-Cox) test. All statistical analyses were performed using GraphPad Prism 7 software. A two-sided $P \leq 0.05$ was considered a statistically significant difference. Data were presented as mean ± SEM.

Results

Characterization of the dose response relationships for ZSJ-0228 in the treatment of LN

To characterize the dose response relationships and optimal dosing levels for ZSJ-0228 in the treatment of LN, ZSJ-022 was administered at 0.5 (L1), 1.0 (L2), 3.0 (L3), and 8.0 (L4) mg/kg/day Dex equivalent. After eight weeks of treatment, 2 out of 14 mice (14%) in the ZSJ-0228 L1 group normalized urine protein levels (Figure 1A). For L2 and L3 groups, 58% of the mice normalized urine protein levels. Unexpectedly, only 31% of the mice in L4 group normalized urine protein levels. Proteinuria was ameliorated in 33% (4 out of 12) of the mice in the free daily Dex group and 8% (1 out of 12) of the mice in the free monthly Dex group. Proteinuria in the saline group not only persisted, but also increased with the disease progression. The average proteinuria level in the ZSJ-0228 L2 group was significantly lower than that in the Dex-daily group ($P < 0.05$) and Dex-monthly group ($P < 0.001$), suggesting that ZSJ-0228 is more effective in ameliorating LN than dose equivalent free Dex. Mice treated with ZSJ-0228 at L2 and L3 dosing levels showed more potent efficacy than those treated at L1 level, consistent with dose-dependency of ZSJ-0228 treatment.

The survival rates in the different groups were consistent with results from the analysis of the proteinuria, *i.e.*, mice with amelioration of proteinuria demonstrated increased survival rates. As shown in Figure 1B, 57%, 92%, 83%, and 69% of the mice treated with ZSJ-0228 at L1, L2, L3, and L4 levels survived over the entire two-month treatment period, respectively. 67% of mice in the daily Dex group and 33% of the mice in the monthly Dex group survived after eight weeks of treatment. Only 2 out of 13 mice (23%) survived to the end of the study in the saline group. The median survival of the saline group was ~five weeks after treatment initiation, which roughly equaled the age of 35 weeks. ZSJ-0228 at L2, L3, and L4 doses and daily Dex treatment significantly increased the survival rate of mice compared with the saline treatment group, providing evidence that ZSJ-0228 and daily Dex increased survival of NZB/W F1 mice with LN. The survival curve in the ZSJ-0228 L1 group was more favorable than the saline group, but did not reach statistically significance, while mice in monthly Dex group had a similar lifespan compared to mice in the saline group.

The effect of ZSJ-0228 dosing levels on renal pathology

The therapeutic efficacy of ZSJ-0228 at different dosing levels was further evaluated by histological analysis of kidney sections. Glomerular hypercellularity, presence of wire-loop lesions, and cellular crescent lesions (Figure 2C) were analyzed in each renal section. The

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

percentage of abnormal glomeruli in renal sections from mice treated with ZSJ-0228 at L1, L2, L3, and L4 levels were 76%, 51%, 52%, and 63%, respectively (Figure 2A), indicating that dosing levels of ZSJ-0228 at L2 and L3 were most effective in resolving glomerular pathology. For daily Dex, monthly Dex and saline controls, the percentages of abnormal glomeruli were 55%, 83%, and 82%, respectively. The frequency of abnormal glomeruli in the ZSJ-0228 treatment at L2, L3, L4 dosing levels and daily Dex treatment group were significantly lower than in the saline group. Monthly Dex has no impact on glomerular lesions. These results were reflected in the kidney histological scores (Figure 2B) and representative PAS-stained kidney sections (Figure 2C).

ZSJ-0228 does not inhibit anti-dsDNA IgG or renal immune complex deposition

Serum anti-dsDNA IgG levels were analyzed to assess the potential dose-dependent effect of ZSJ-0228 on serum anti-dsDNA IgG levels based on evidence that anti-dsDNA IgG plays a pivotal role in LN development.[26] At the pretreatment time point, the average anti-dsDNA IgG levels were ~400 kU/mL in all groups with LN (Figure 3A). Similar to the saline group, the anti-dsDNA IgG levels did not change over the time course of the study in the ZSJ-0228 treated mice at all dosing levels and in the monthly Dex group. In contrast, daily Dex treatment resulted in a significant decrease in anti-dsDNA IgG levels by the end of the study ($P < 0.05$). These data suggest that the efficacy of ZSJ-0228 in ameliorating nephritis is not related to an effect of the treatment on anti-dsDNA IgG levels but is dependent on alternate mechanism(s) that differ from the effects of daily Dex treatment and is independent of autoantibody inhibition.

Next, we explored the effects of different treatments on renal immune complex deposition. Extensive glomerular immune complex deposition was detected in the saline group (Figure 3B, C). The immune complex deposition was also detected in the ZSJ-0228 treated mice at all dosing levels. Quantitative analysis of the staining pattern revealed that there was no statistically significant difference between the extent of immune complex deposition in any of the ZSJ-0228 groups or the saline group (Figure 3B), indicating that the beneficial effects of ZSJ-0228 are independent of effects on immune complex deposition. In the daily Dex group, the level of renal immune complex deposition was significantly lower than that in the monthly Dex or saline groups, indicating daily Dex treatment does reduce kidney immune complex deposition. Different from Dex-daily treatment, monthly Dex treatment did not have any impact on renal immune complex deposition.

Dose dependent effects of ZSJ-0228 treatment on renal macrophage infiltration

There is good evidence that in addition to the role of immune complex deposition that recruitment and activation of inflammatory cells, including macrophages, play a key role in the pathogenesis of LN.[27, 28] To assess the effects of the different treatments on renal macrophage glomerular macrophage infiltration, renal sections were examined by immunohistochemical staining using fluorescent labeled rat anti-mouse F4/80 antibody (eFluor 570 (eBioscience)) antibodies. As shown in Figure 4A, abundant macrophage infiltration was found in the saline group. Compared to the saline group, macrophage infiltration levels were slightly lower in the ZSJ-0228 L1 group and ZSJ-0228 L4 group but changes did not reach significant difference. In contrast, macrophage infiltration in the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

ZSJ-0228 treated mice at L2 and L3 levels was significantly lower than the saline group. These data suggest that ZSJ-0228 may in part attenuate nephritis by inhibiting macrophage infiltration and that the inhibitory effect is dose-dependent. The macrophage infiltration level of the daily Dex group was also lower than the saline group, but the changes were not statistically significantly different. Similar to the saline group, extensive macrophage infiltration was observed in the kidneys of mice in the monthly Dex group. Quantitative analysis of the macrophage infiltration is presented in Figure 4B.

Assessment of the effects of ZSJ-0228 on GC-associated toxicities

Over the 8-week treatment study, no significant body weight loss was observed in any of the ZSJ-0228 groups (Figure 5A). Chronic GC administration induces hypothalamic-pituitary-adrenal axis suppression and adrenal gland atrophy.[29] To assess the dose-dependent effects of ZSJ-0228, the weight of the adrenal glands was assessed at necropsy. As shown in Figure 5B, the average adrenal gland weight of the mice in the daily Dex group was significantly lower than the weight in the saline treated mice. In contrast, similar to the saline treated animals, ZSJ-0228 treatment at all dosing levels did not affect adrenal gland weight, indicating that ZSJ-0228 treatment does not induce adrenal gland suppression, even at a very high dose (8 mg/kg/day Dex equivalent). Monthly pulse administration of free Dex did not affect adrenal gland weight as shown in Figure 5B.

Splenomegaly frequently occurs in lupus-prone mouse strains, including NZB/W F1 mice.[30] We next examined the effects of the treatments on spleen weight. The average spleen weights of the mice in the ZSJ-0228 groups and monthly Dex group were similar to that of the saline group (Figure 5C), suggesting that ZSJ-0228 and monthly Dex treatments did not prevent the development of splenomegaly. The spleen weights in the daily Dex group were significantly lower than those in all other groups with an average weight of ~0.075 g, consistent with results from previous studies.[31–34]

We measured the serum total IgG levels in mice from the different groups. Prior to the treatment, there was no statistically significant difference in the serum total IgG level in any of the groups (Figure 5D). At the end of the study, the serum IgG level in the ZSJ-0228 treated groups and the monthly Dex group were similar to the levels in the saline group. In contrast, serum IgG levels were significantly reduced in the daily Dex group at the 4-week time point ($P < 0.05$) and were further reduced at the 8-week time point ($P < 0.0001$). In addition to the serum total IgG levels, we also measured peripheral white blood cell (WBC) counts at the end of the study. As shown in Figure 5E, there was no significant difference among all ZSJ-0228 groups, the monthly Dex group, and the saline group. However, WBC counts in the daily Dex group were significantly lower compared to the other groups ($P < 0.001$).

To investigate the impact of the different treatments on blood glucose levels, we measured blood glucose levels every four weeks. Interestingly, rather than increasing blood glucose levels, daily Dex treatment significantly decreased blood glucose levels after 4-week treatment ($P < 0.05$) and 8-week treatment ($P < 0.01$) compared to baseline levels. The mean blood glucose levels of mice in the daily Dex group were also significantly lower than the levels in all other groups ($P < 0.01$, Figure 5F), consistent with an effect of Dex

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

on glucose metabolism. There was no significant difference in mean blood glucose levels among ZSJ-0228 groups, Dex-monthly group, and saline group, consistent with an absence of an effect of the ZSJ-0228 treatments on blood glucose metabolism.

Effect of ZSJ-0228 on bone density and quality

Osteoporosis is among the major toxicities associated with chronic GC administration. Bone loss occurs early after the initiation of GC therapy and is related to the dosage and treatment duration.[35, 36] To explore the impact of ZSJ-0228 on bone density and quality, the distal femurs of mice from the different treatment groups were scanned and analyzed. The mean bone mineral density (BMD) was the lowest in the daily Dex treated mice, and it was significantly lower than the density in two of the ZSJ-0228 treatment groups (L1, $P < 0.01$; L2, $P < 0.05$, Figure 6A). No significant difference was observed among all ZSJ-0228 groups and the saline group. The bone volume/tissue volume ratio (BV/TV) and trabecular thickness were also lowest in the daily Dex group (Figure 6B, C). The BV/TV of the daily Dex group was significantly lower than two ZSJ-0228 treatment groups (L1, $P < 0.05$; L2, $P < 0.05$). There was no significant difference among all groups in trabecular number (Figure 6D). The results are consistent with an absence of an effect of ZSJ-0228 treatment on bone mass or quality.

Discussion

The development of new therapeutic agents to improve the outcomes and reduce the associated toxicities associated with the treatment of LN has been a very challenging process. Over the last 70 years, only belimumab and voclosporin have been approved by the US FDA, and GCs remain an essential component of the clinical treatment of LN related to their potent anti-inflammatory and immunosuppressive effects, and the lack of alternative therapies.[6, 7] To reduce the adverse effects of chronic GC therapy, GC formulations have been developed with the goal of improving the PK/BD profiles. Several formulations have been shown to reduce pathological inflammation in rodent models of inflammatory arthritis,[37, 38] although the improvements in therapeutic efficacy were not associated with a significant reduction in associated systemic GC toxicity. We have developed a macromolecular prodrug nanomedicine of dexamethasone, ZSJ-0228, and demonstrated its efficacy in ameliorating established LN in lupus-prone NZB/W F1 mice, while avoiding GC-associated adverse effects.[20] The present studies were undertaken to establish the dose response relationships for ZSJ-0228 to confirm its' efficacy and safety, with the goal of identifying the optimal GC formulation.

All of the drugs or vehicles (except for daily Dex) were administered intravenously, as this delivery route offers the highest bioavailability.[39] Daily Dex was administered i.p. because it is impractical to do daily repetitive i.v. injections on a single tail for eight weeks. In rodent animal models, most substances administered into peritoneal cavity can be very quickly absorbed. Therefore, it is generally considered that the systemic exposure (AUC and C_{max}) of an i.p.-administered substance is close to that of an i.v. route, and i.p. injection in mice is an acceptable replacement for i.v. injection in related pharmacological studies where the goal is to evaluate efficacy.[40] Therefore, no adjustment was made to daily Dex i.p. dosing

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

level. After eight weeks of treatment, the efficacy of ZSJ-0228 in ameliorating LN in female NZB/W F1 mice was dose-dependent, where the L2 and L3 doses were more effective than the L1 dose. The L3 dose of ZSJ-0228 displayed similar therapeutic efficacy compared to the L2 dose, indicating ZSJ-0228 at the L2 dose may have achieved maximal efficacy. Interestingly, the L4 dose of ZSJ-0228 was associated with reduced efficacy compared to the L2 or L3 dosing levels of ZSJ-0228. We speculate that the high micelle content of the L4 ZSJ-0228 formulation may be associated with renal toxicity following i.v. injection, since exposure to nanoparticles has the potential to induce glomerular swelling, membrane thickening and degeneration, and renal tubular cell necrosis.[41] Since the renal pathology associated with nanoparticles is similar to the LN lesion, it is difficult to test this hypothesis. Alternatively, the reduced efficacy of the L4 formulation could be related to the toxicity of PEG, which is a component of all of the ZSJ-0228 formulations and can be released *in vivo*. PEG itself has been associated with organ toxicity at very high parenteral doses, and the kidney is the route of excretion for unmodified PEG. Renal PEG toxicity is associated with vacuolation of the proximal kidney tubule epithelial cells.[42] However, no proximal tubule vacuolation was detected in any of the kidney sections from the different ZSJ-0228 groups (data not shown), suggesting that the compromised efficacy of the L4 dose ZSJ-0228 was not related to PEG toxicity. As a third possibility, we posit that due to the increased concentration of ZSJ-0228, the L4 formulation has a micelle/aggregation of larger size, which may shift the biodistribution pattern of ZSJ-0228, with increased uptake in the liver and spleen and reduced distribution to the kidney, leading to reduced efficacy of the ZSJ-0228 in the treatment of the LN. While dynamic light scattering results confirmed the increase of ZSJ-0228 micelle/aggregation size (from 40 to ~ 1,000 nm) with increased concentration, optical imaging results (data not shown) do not support an altered biodistribution pattern. Additional studies are needed to define the mechanism(s) responsible for the optimal ZSJ-0228 dosing range of 1.0 – 3.0 mg/kg/day (Dex eq.).

Consistent with our previous study,[21] monthly Dex treatment did not result in a therapeutic effect compared to saline administration, indicating that additional dosing following pulse GC is needed. Daily Dex treatment was less efficacious in reducing renal pathology than dose equivalent ZSJ-0228 L2 treatment. However, daily Dex treatment resulted in a reduction in serum anti-dsDNA IgG levels consistent with results from other studies.[43, 44]

Chronic GC treatment is associated with multiple serious side effects, including osteoporosis, increased susceptibility to infections, adrenal gland atrophy, impaired glucose metabolism,[9, 10] and these adverse effects contribute significantly to morbidity in patients with LN.[45] Here, we show that daily Dex resulted in significant GC-associated adverse effects, including the interference with glucose metabolism.[46–48] Contrary to observations in humans, daily Dex treatment significantly decreased the blood glucose level in NZB/W F1 mice (Figure 5F), an observation that has also been reported in other studies.[49, 50] Boortz *et al*. demonstrated the complexity of GCs' physiology by showing that GCs can enhance insulin secretion *in vivo* beyond the level required to counteract insulin resistance, thereby leading to reduced blood glucose levels.[50] We hypothesize that the nephrotropicity of ZSJ-0228 not only plays a key role in its' superior efficacy but also contributes to its' excellent safety profile with all of the ZSJ-0228 formulations, including L4 (Figure 5 and 6).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Conclusions

The present dose escalation study was performed to further establish the therapeutic efficacy and safety of a PEG-based micelle-forming dexamethasone prodrug (ZSJ-0228) in the treatment of LN in NZB/W F1 mice. The most effective dose of ZSJ-0228 was between 1.0 and 3.0 mg/kg/day Dex equivalent. Importantly, ZSJ-0228 treatment did not induce GC-associated adverse effects, even at the highest dose level tested. The findings from this study provide additional information regarding the optimal ZSJ-0228 dosing level for future clinical translation into humans. In addition, ZSJ-0228 may have utility in the management of other kidney diseases, including focal segmental glomerulosclerosis, and in kidney transplantation, where GCs are regularly applied as first-line therapy.

Acknowledgments

This study was supported, in part, by the National Institute of Allergy and Infectious Diseases of the United States National Institutes of Health (R01 AI119090) and UNMC College of Pharmacy. X. Xu received a scholarship from China Scholarship Council (201907067014). The content is solely the authors' responsibility and does not necessarily represent the official views of the National Institutes of Health.

References

1. Moulton VR; Suarez-Fueyo A; Meidan E; Li H; Mizui M; Tsokos GC Pathogenesis of Human Systemic Lupus Erythematosus: A Cellular Perspective. Trends Mol Med 2017, 23, (7), 615–635. [PubMed: 28623084]
2. Durcan L; O'Dwyer T; Petri M Management strategies and future directions for systemic lupus erythematosus in adults. Lancet 2019, 393, (10188), 2332–2343. [PubMed: 31180030]
3. Ocampo-Piraquive V; Nieto-Aristizabal I; Canas CA; Tobon GJ Mortality in systemic lupus erythematosus: causes, predictors and interventions. Expert Rev Clin Immunol 2018, 14, (12), 1043–1053. [PubMed: 30338717]
4. Almaani S; Meara A; Rovin BH Update on Lupus Nephritis. Clin J Am Soc Nephrol 2017, 12, (5), 825–835. [PubMed: 27821390]
5. Parikh SV; Almaani S; Brodsky S; Rovin BH Update on Lupus Nephritis: Core Curriculum 2020. Am J Kidney Dis 2020, 76, (2), 265–281. [PubMed: 32220510]
6. Bertsias GK; Tektonidou M; Amoura Z; Aringer M; Bajema I; Berden JH; Boletis J; Cervera R; Dorner T; Doria A; Ferrario F; Floege J; Houssiau FA; Ioannidis JP; Isenberg DA; Kallenberg CG; Lightstone L; Marks SD; Martini A; Moroni G; Neumann I; Praga M; Schneider M; Starra A; Tesar V; Vasconcelos C; van Vollenhoven RF; Zakharova H; Haubitz M; Gordon C; Jayne D; Boumpas DT; European League Against R; European Renal Association-European D; Transplant A Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. Ann Rheum Dis 2012, 71, (11), 1771–82. [PubMed: 22851469]
7. Hahn BH; McMahon MA; Wilkinson A; Wallace WD; Daikh DI; Fitzgerald JD; Karpouzas GA; Merrill JT; Wallace DJ; Yazdany J; Ramsey-Goldman R; Singh K; Khalighi M; Choi SI; Gogia M; Kafaja S; Kamgar M; Lau C; Martin WJ; Parikh S; Peng J; Rastogi A; Chen W; Grossman JM; American College of, R. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. Arthritis Care Res (Hoboken) 2012, 64, (6), 797–808. [PubMed: 22556106]
8. Rice JB; White AG; Scarpati LM; Wan G; Nelson WW Long-term Systemic Corticosteroid Exposure: A Systematic Literature Review. Clin Ther 2017, 39, (11), 2216–2229. [PubMed: 29055500]
9. Schacke H; Docke WD; Asadullah K Mechanisms involved in the side effects of glucocorticoids. Pharmacol Ther 2002, 96, (1), 23–43. [PubMed: 12441176]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

10. Oray M; Abu Samra K; Ebrahimiadib N; Meese H; Foster CS Long-term side effects of glucocorticoids. Expert Opin Drug Saf 2016, 15, (4), 457–65. [PubMed: 26789102]
11. Hiraki LT; Benseler SM; Tyrrell PN; Hebert D; Harvey E; Silverman ED Clinical and laboratory characteristics and long-term outcome of pediatric systemic lupus erythematosus: a longitudinal study. J Pediatr 2008, 152, (4), 550–6. [PubMed: 18346514]
12. Brunner HI; Gladman DD; Ibanez D; Urowitz MD; Silverman ED Difference in disease features between childhood-onset and adult-onset systemic lupus erythematosus. Arthritis Rheum 2008, 58, (2), 556–62. [PubMed: 18240232]
13. Adcock IM; Nasuhara Y; Stevens DA; Barnes PJ Ligand-induced differentiation of glucocorticoid receptor (GR) trans-repression and transactivation: preferential targetting of NF-kappaB and lack of I-kappaB involvement. Br J Pharmacol 1999, 127, (4), 1003–11. [PubMed: 10433509]
14. Newton R; Holden NS Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? Mol Pharmacol 2007, 72, (4), 799–809. [PubMed: 17622575]
15. Buckbinder L; Robinson RP The glucocorticoid receptor: molecular mechanism and new therapeutic opportunities. Curr Drug Targets Inflamm Allergy 2002, 1, (2), 127–36. [PubMed: 14561195]
16. Kym PR; Kort ME; Coghlan MJ; Moore JL; Tang R; Ratajczyk JD; Larson DP; Elmore SW; Pratt JK; Stashko MA; Falls HD; Lin CW; Nakane M; Miller L; Tyree CM; Miner JN; Jacobson PB; Wilcox DM; Nguyen P; Lane BC Nonsteroidal selective glucocorticoid modulators: the effect of C-10 substitution on receptor selectivity and functional potency of 5-allyl-2,5-dihydro-2,2,4-trimethyl-1H-[1]benzopyrano[3,4-f]quinolines. J Med Chem 2003, 46, (6), 1016–30. [PubMed: 12620078]
17. Ayroldi E; Macchiarulo A; Riccardi C Targeting glucocorticoid side effects: selective glucocorticoid receptor modulator or glucocorticoid-induced leucine zipper? A perspective. FASEB J 2014, 28, (12), 5055–70. [PubMed: 25205742]
18. Harris ED Jr.; Emkey RD; Nichols JE; Newberg A Low dose prednisone therapy in rheumatoid arthritis: a double blind study. J Rheumatol 1983, 10, (5), 713–21. [PubMed: 6358491]
19. Saag KG; Koehnke R; Caldwell JR; Brasington R; Burmeister LF; Zimmerman B; Kohler JA; Furst DE Low dose long-term corticosteroid therapy in rheumatoid arthritis: an analysis of serious adverse events. Am J Med 1994, 96, (2), 115–23. [PubMed: 8109596]
20. Jia Z; Wang X; Wei X; Zhao G; Foster KW; Qiu F; Gao Y; Yuan F; Yu F; Thiele GM; Bronich TK; O'Dell JR; Wang D Micelle-Forming Dexamethasone Prodrug Attenuates Nephritis in Lupus-Prone Mice without Apparent Glucocorticoid Side Effects. ACS Nano 2018, 12, (8), 7663–7681. [PubMed: 29965725]
21. Zhao Z; Jia Z; Foster KW; Wei X; Qiao F; Jiang H; Jin Y; Li G; Chen N; Zhao G; Thiele GM; Medlin JL; O'Dell JR; Wang D Dexamethasone prodrug nanomedicine (ZSJ-0228) treatment significantly reduces lupus nephritis in mice without measurable side effects - A 5-month study. Nanomedicine 2020, 102302. [PubMed: 32980548]
22. Liu D; Ahmet A; Ward L; Krishnamoorthy P; Mandelcorn ED; Leigh R; Brown JP; Cohen A; Kim H A practical guide to the monitoring and management of the complications of systemic corticosteroid therapy. Allergy Asthma Clin Immunol 2013, 9, (1), 30. [PubMed: 23947590]
23. Hua C; Buttgereit F; Combe B Glucocorticoids in rheumatoid arthritis: current status and future studies. RMD Open 2020, 6, (1).
24. Bynote KK; Hackenberg JM; Korach KS; Lubahn DB; Lane PH; Gould KA Estrogen receptor-alpha deficiency attenuates autoimmune disease in (NZB x NZW)F1 mice. Genes Immun 2008, 9, (2), 137–52. [PubMed: 18200028]
25. Yuan F; Nelson RK; Tabor DE; Zhang Y; Akhter MP; Gould KA; Wang D Dexamethasone prodrug treatment prevents nephritis in lupus-prone (NZB x NZW)F1 mice without causing systemic side effects. Arthritis Rheum 2012, 64, (12), 4029–39. [PubMed: 22886616]
26. Yung S; Chan TM Autoantibodies and resident renal cells in the pathogenesis of lupus nephritis: getting to know the unknown. Clin Dev Immunol 2012, 2012, 139365. [PubMed: 22761629]
27. Schiffer L; Bethunaickan R; Ramanujam M; Huang W; Schiffer M; Tao H; Madaio MP; Bottinger EP; Davidson A Activated renal macrophages are markers of disease onset and disease remission in lupus nephritis. J Immunol 2008, 180, (3), 1938–47. [PubMed: 18209092]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

28. Ma R; Jiang W; Li Z; Sun Y; Wei Z Intrarenal macrophage infiltration induced by T cells is associated with podocyte injury in lupus nephritis patients. Lupus 2016, 25, (14), 1577–1586. [PubMed: 27147620]
29. Karangizi AHK; Al-Shaghana M; Logan S; Criseno S; Webster R; Boelaert K; Hewins P; Harper L Glucocorticoid induced adrenal insufficiency is common in steroid treated glomerular diseases - proposed strategy for screening and management. BMC Nephrol 2019, 20, (1), 154. [PubMed: 31060510]
30. Kono DH; Burlingame RW; Owens DG; Kuramochi A; Balderas RS; Balomenos D; Theofilopoulos AN Lupus susceptibility loci in New Zealand mice. Proc Natl Acad Sci U S A 1994, 91, (21), 10168–72. [PubMed: 7937857]
31. Reber LL; Daubeuf F; Plantinga M; De Cauwer L; Gerlo S; Waelput W; Van Calenbergh S; Tavernier J; Haegeman G; Lambrecht BN; Frossard N; De Bosscher K A dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a mouse model of asthma. J Immunol 2012, 188, (7), 3478–87. [PubMed: 22393156]
32. Graversen JH; Svendsen P; Dagnaes-Hansen F; Dal J; Anton G; Etzerodt A; Petersen MD; Christensen PA; Moller HJ; Moestrup SK Targeting the hemoglobin scavenger receptor CD163 in macrophages highly increases the anti-inflammatory potency of dexamethasone. Mol Ther 2012, 20, (8), 1550–8. [PubMed: 22643864]
33. Mushaben EM; Brandt EB; Hershey GK; Le Cras TD Differential effects of rapamycin and dexamethasone in mouse models of established allergic asthma. PLoS One 2013, 8, (1), e54426. [PubMed: 23349887]
34. Grahnemo L; Jochems C; Andersson A; Engdahl C; Ohlsson C; Islander U; Carlsten H Possible role of lymphocytes in glucocorticoid-induced increase in trabecular bone mineral density. J Endocrinol 2015, 224, (1), 97–108. [PubMed: 25359897]
35. Canalis E; Delany AM Mechanisms of glucocorticoid action in bone. Ann N Y Acad Sci 2002, 966, 73–81. [PubMed: 12114261]
36. Briot K; Roux C Glucocorticoid-induced osteoporosis. RMD Open 2015, 1, (1), e000014. [PubMed: 26509049]
37. Metselaar JM; Wauben MH; Wagenaar-Hilbers JP; Boerman OC; Storm G Complete remission of experimental arthritis by joint targeting of glucocorticoids with long-circulating liposomes. Arthritis Rheum 2003, 48, (7), 2059–66. [PubMed: 12847701]
38. Quan L; Zhang Y; Crielaard BJ; Dusad A; Lele SM; Rijcken CJF; Metselaar JM; Kostkova H; Etrych T; Ulbrich K; Kiessling F; Mikuls TR; Hennink WE; Storm G; Lammers T; Wang D Nanomedicines for inflammatory arthritis: head-to-head comparison of glucocorticoid-containing polymers, micelles, and liposomes. ACS Nano 2014, 8, (1), 458–466. [PubMed: 24341611]
39. Hirota J; Shimizu S Routes of administration. The laboratory mouse 2012, 2, 709–725.
40. Al Shoyaib A; Archie SR; Karamyan VT Intraperitoneal Route of Drug Administration: Should it Be Used in Experimental Animal Studies? Pharm Res 2019, 37, (1), 12. [PubMed: 31873819]
41. Iavicoli I; Fontana L; Nordberg G The effects of nanoparticles on the renal system. Crit Rev Toxicol 2016, 46, (6), 490–560. [PubMed: 27195425]
42. Rudmann DG; Alston JT; Hanson JC; Heidel S High molecular weight polyethylene glycol cellular distribution and PEG-associated cytoplasmic vacuolation is molecular weight dependent and does not require conjugation to proteins. Toxicol Pathol 2013, 41, (7), 970–83. [PubMed: 23788571]
43. Spronk PE; Horst G; Van Der Gun BT; Limburg PC; Kallenberg CG Anti-dsDNA production coincides with concurrent B and T cell activation during development of active disease in systemic lupus erythematosus (SLE). Clin Exp Immunol 1996, 104, (3), 446–53. [PubMed: 9099929]
44. Dean GS; Tyrrell-Price J; Crawley E; Isenberg DA Cytokines and systemic lupus erythematosus. Ann Rheum Dis 2000, 59, (4), 243–51. [PubMed: 10733469]
45. Ruiz-Irastorza G; Danza A; Khamashta M Glucocorticoid use and abuse in SLE. Rheumatology (Oxford) 2012, 51, (7), 1145–53. [PubMed: 22271756]
46. Clore JN; Thurby-Hay L Glucocorticoid-induced hyperglycemia. Endocr Pract 2009, 15, (5), 469–74. [PubMed: 19454391]
47. Hwang JL; Weiss RE Steroid-induced diabetes: a clinical and molecular approach to understanding and treatment. Diabetes Metab Res Rev 2014, 30, (2), 96–102. [PubMed: 24123849]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

48. Perez A; Jansen-Chaparro S; Saigi I; Bernal-Lopez MR; Minambres I; Gomez-Huelgas R Glucocorticoid-induced hyperglycemia. J Diabetes 2014, 6, (1), 9–20. [PubMed: 24103089]

49. Cummings BP; Bremer AA; Kieffer TJ; D'Alessio D; Havel PJ Investigation of the mechanisms contributing to the compensatory increase in insulin secretion during dexamethasone-induced insulin resistance in rhesus macaques. J Endocrinol 2013, 216, (2), 207–15. [PubMed: 23151361]

50. Boortz KA; Syring KE; Lee RA; Dai C; Oeser JK; McGuinness OP; Wang JC; O'Brien RM G6PC2 Modulates the Effects of Dexamethasone on Fasting Blood Glucose and Glucose Tolerance. Endocrinology 2016, 157, (11), 4133–4145. [PubMed: 27653037]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

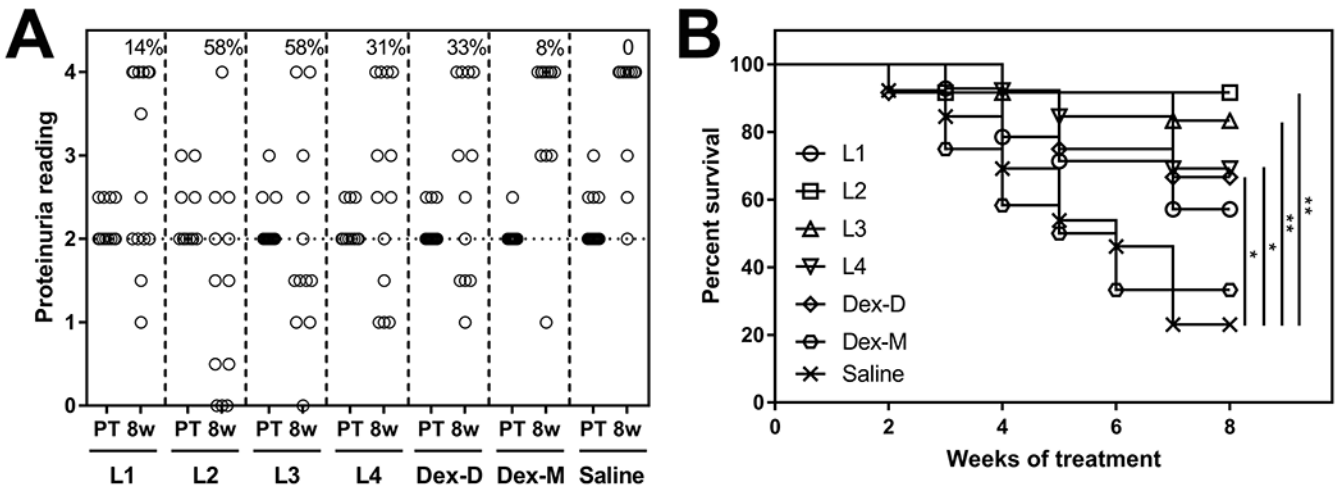


Figure 1.

ZSJ-0228 ameliorates proteinuria and expands NZB/W F1 mice lifespan. **(A)** Proteinuria readings in mice in the different treatment groups: ZSJ-0228 L1 dose group (0.5 mg/kg/day Dex eq., n=14), L2 dose group (1.0 mg/kg/day Dex eq., n=12), L3 dose group (3.0 mg/kg/day Dex eq., n=12), L4 dose group (8.0 mg/kg/day Dex eq., n=13); daily Dex group (Dex-D, 1.0 mg/kg/day, n=12); monthly Dex group (Dex-M, 1.0 mg/kg/day Dex eq., n=12); and saline group (n=13) at pretreatment (PT) and treatment end points (8w). The ratio of mice with proteinuria resolution (proteinuria reading < 2) at the end point is displayed at the top right corner of each panel. For mice that did not survive to the end of the treatments, the proteinuria readings were labeled as 4 at the 8-week time point. **(B)** Kaplan-Meier survival curve for each group. Log-rank (Mantel-Cox) test. * $P < 0.05$, ** $P < 0.01$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

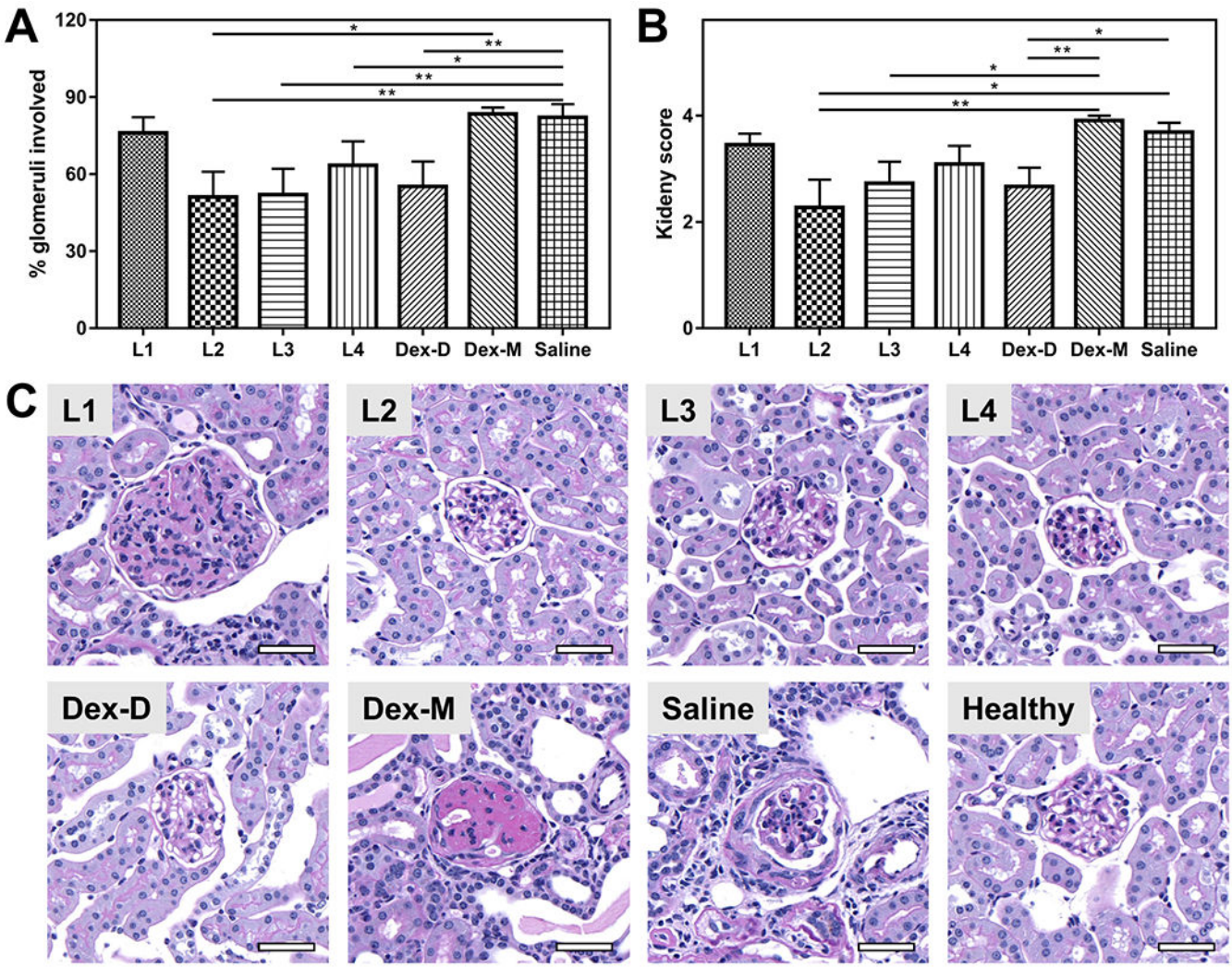


Figure 2.

ZSJ-0228 attenuates LN in NZB/W F1 mice. **(A)** The average percentage of abnormal glomeruli demonstrating glomerular hypercellularity, wire-loop lesions and cellular crescent lesions in each group. **(B)** Average histological score for each group. **(C)** Representative PAS-stained slides from each group. A representative renal section from a young NZB/W F1 mouse (16 weeks old) was used to demonstrate the renal histology in mice without nephritis. Scale bar: 50 μm. Results are presented as mean ± SEM. One-way ANOVA with post hoc Tukey's test. * $P < 0.05$, ** $P < 0.01$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

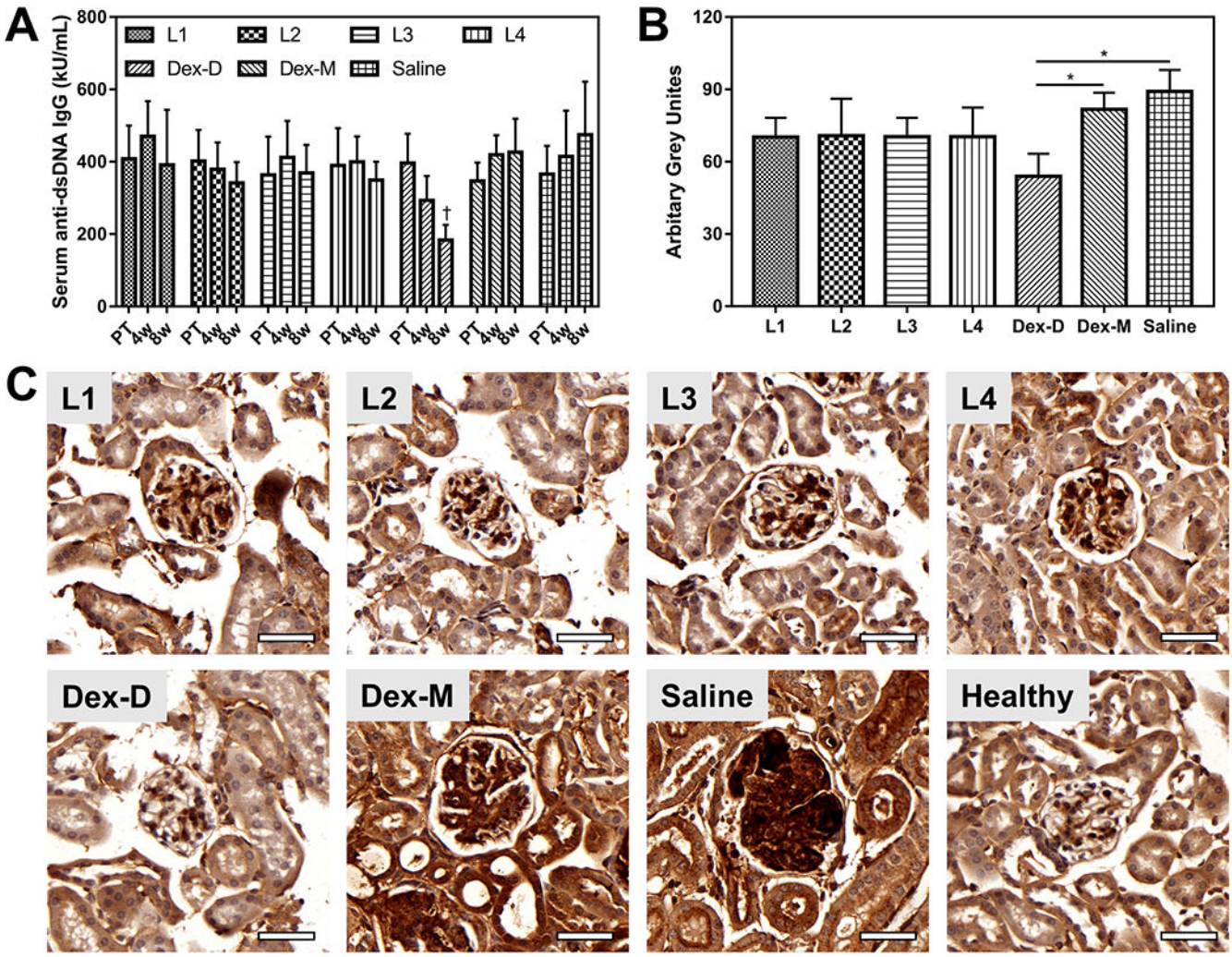


Figure 3.

Treatment effects on anti-dsDNA IgG levels and kidney immune complex deposition levels. **(A)** Average serum anti-dsDNA IgG levels in each group at pretreatment (PT) time point, 4-week (4w) time point, and 8-week (8w) time point, measured by ELISA assay. At each time point, only mice that survived were available for serum analysis. The dagger symbol (†) highlights the significant difference in the staining pattern between the labeled time point and the pretreatment time point. **(B)** Quantification of renal immune complex deposition staining. **(C)** Immunohistochemistry immune complex staining in representative kidney sections from each group. Scale bar: 50 μm. A young NZB/W F1 mouse (16 weeks old) was used to demonstrate the staining pattern in healthy mice. Results are presented as mean ± SEM. Wilcoxon matched-pairs signed rank test. † $P < 0.05$. One-way ANOVA with post hoc Tukey's test. * $P < 0.05$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

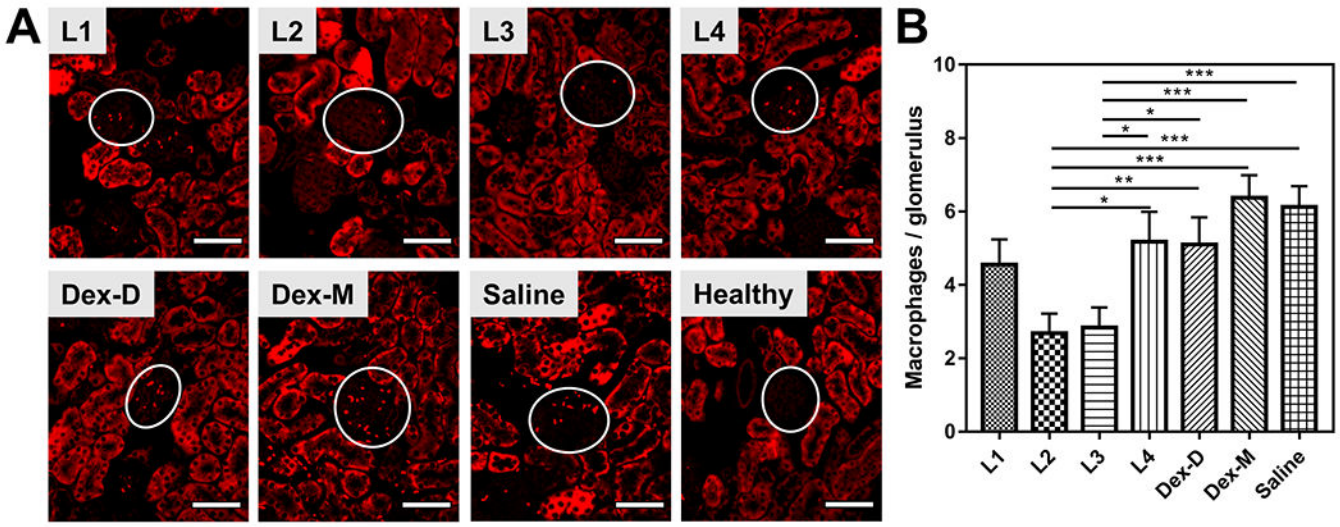


Figure 4.

Impact of the treatments on renal macrophage infiltration. **(A)** Representative kidney sections, stained by immunofluorescence, from the different groups. The white circles represent glomeruli. Scale bar = 50 μm. Sections from a young NZB/W F1 mouse (16 weeks old) was used to illustrate the pattern of kidney macrophage infiltration in healthy kidneys. **(B)** Quantitative analysis of glomerular macrophage infiltration in the different treatment groups. Results are presented by mean ± SEM. One-way ANOVA with post hoc Tukey's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

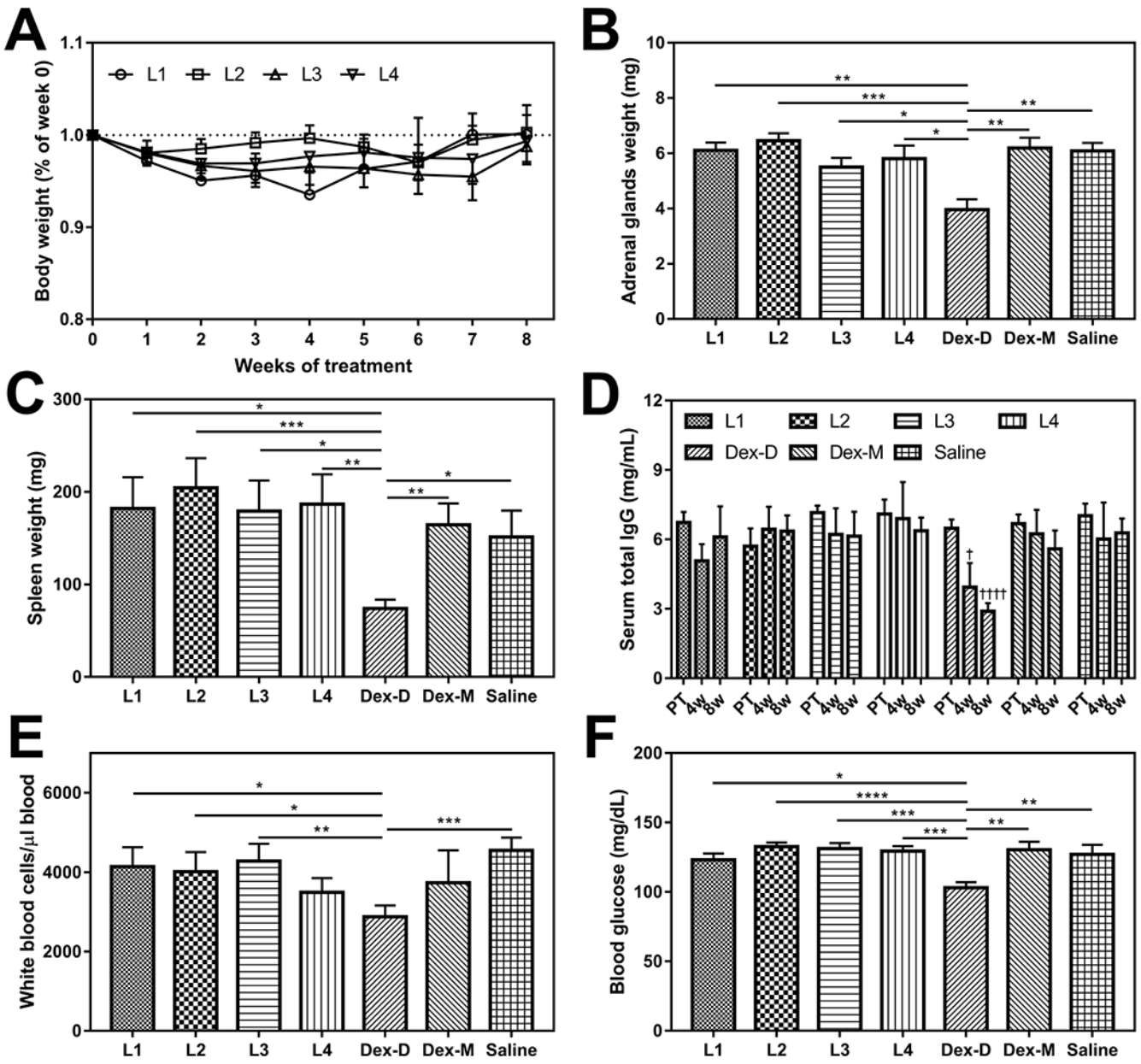


Figure 5.

Toxicity studies. **(A)** Body weight changes in the different treatment groups. **(B)** Average adrenal gland weight in the different treatment groups. **(C)** Average spleen weight in the different treatment groups, determined at the time of sacrifice. **(D)** Total serum IgG levels, determined at the pretreatment time point (PT), 4-week time point (4w), and 8-week time point (8w). The dagger symbol (†) demonstrated the significant difference between the labeled time point and pretreatment time point. **(E)** Peripheral white blood cell counts at the end of the study. **(F)** Blood glucose levels at the end time point. Results are presented as mean ± SEM. Wilcoxon matched-pairs signed rank test. † $P < 0.05$, †††† $P < 0.0001$. One-way ANOVA with post hoc Tukey's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

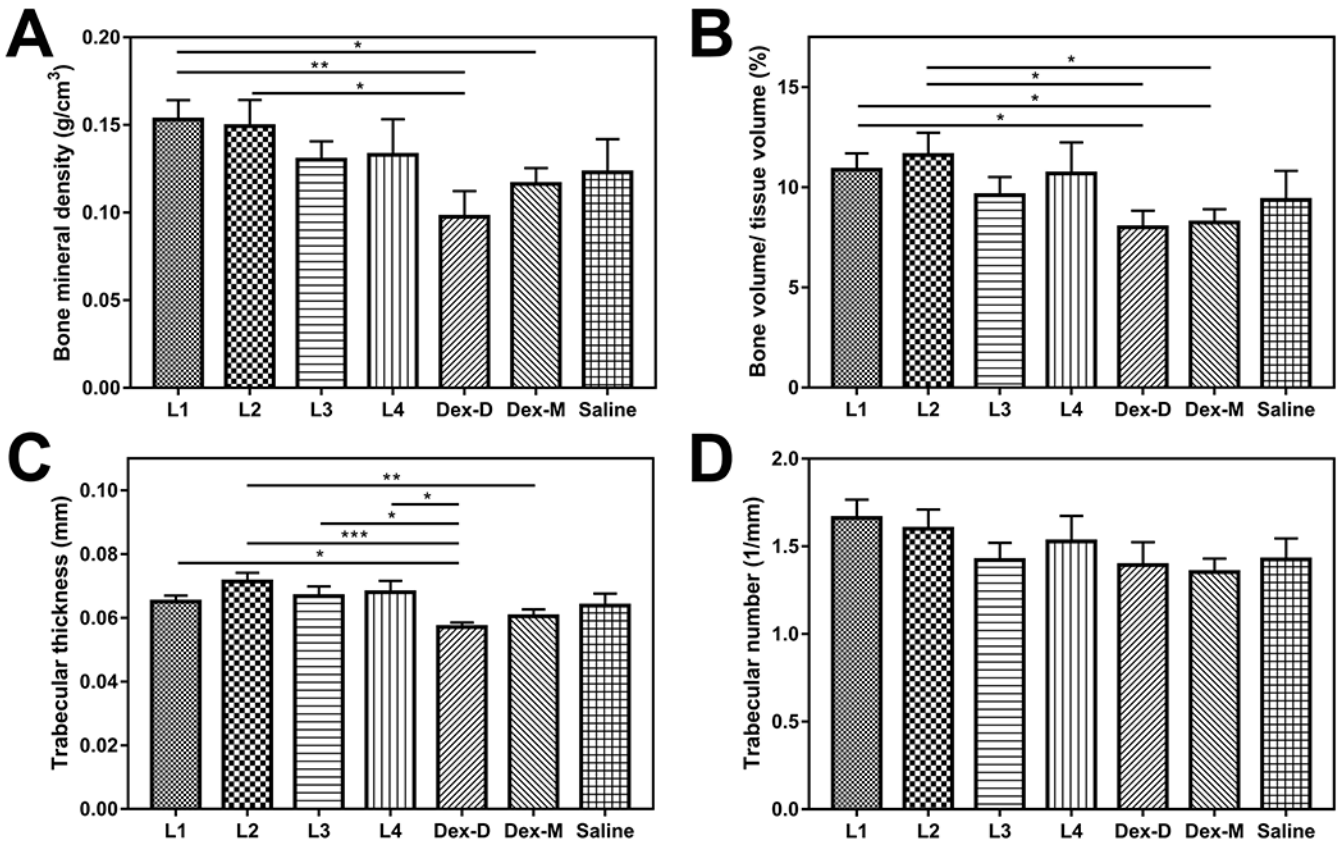


Figure 6.

Impact of ZSJ-0228, monthly Dex, daily Dex, and saline on bone density and quality.

(A) Bone mineral density (BMD). **(B)** bone volume to tissue volume ratio (BV/TV). **(C)** Trabecular thickness (Tr.Th). **(D)** Trabecular number (Tr.N). Results are presented as mean ± SEM. One-way ANOVA with post hoc Tukey's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.