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Head-to-head Comparative Pharmacokinetic and Biodistribution (PK/BD) Study of Two Dexamethasone Prodrug Nanomedicines on Lupus-prone NZB/W F1 Mice

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Abstract

HPMA copolymer-based dexamethasone prodrug (P-Dex) and PEG-based dexamethasone prodrug (PEG-Dex, ZSJ-0228) were previously found to passively target the inflamed kidney and provide potent and sustained resolution of nephritis in NZB/W F1 lupus-prone mice. While both prodrug nanomedicines effectively ameliorate lupus nephritis, they have demonstrated distinctively different safety profiles. To explore the underlining mechanisms of these differences, we conducted a head-to-head comparative PK/BD study of P-Dex and PEG-Dex on NZB/W F1 mice. Overall, the systemic organ/tissue exposures to P-Dex and Dex released from P-Dex were found to be significantly higher than those of PEG-Dex. The high prodrug concentrations were sustained in kidney for only 24 hours, which cannot explain their lasting therapeutic efficacy (> 1 month). P-

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CONFLICTS OF INTERESTS: DW, ZSJ and XBW are co-inventors of the patent (PCT/US16/61728), which covers ZSJ-0228 prodrug technology. DW is a co-founder of Shannon Pharmaceuticals, which has licensed the technology for preclinical and clinical development. Shannon provides DW's laboratory with funding for Chemistry Manufacturing and Controls (CMC) development.

Dex showed sustained presence in liver, spleen and adrenal gland, while the presence of PEG-Dex in these organs was transient. This difference in PK/BD profiles may explain PEG-Dex' superior safety than P-Dex.

Graphical Abstract

When administered systemically, both P-Dex and PEG-Dex showed strong nephrotropism, leading to potent and sustained (> 1 month) resolution of nephritis in NZB/W F1 lupus-prone mice. In this PK/BD study, we found that their high renal concentration was surprisingly short-lived and maintained for only 24 hours. This unique observation cannot explain the sustained amelioration of lupus nephritis by P-Dex and PEG-Dex, suggesting potentially novel pharmacological mechanisms other than sustained local release of Dex from these prodrug nanomedicines.

Keywords

Prodrug Nanomedicine; Lupus Nephritis; Dexamethasone; P-Dex; ZSJ-0228; Pharmacokinetics and Biodistribution

BACKGROUND

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by overproduction of autoantibodies against nuclear antigens, including double-stranded DNA (dsDNA).^{1, 2} The deposition of anti-dsDNA IgG-containing immune complexes in vital organs/tissues leads to chronic inflammation, structural and functional damages, eventually organ failure and mortality.^{3–5} Glucocorticoids (GC) have been used as mainstream treatment for SLE and other rheumatic diseases for more than five decades.⁶ GC bind to the glucocorticoid receptor (GR) which leads to indirect inhibition of pro-inflammatory genes expression (transrepression).^{7, 8} GC's indirect activation of certain gene expression (transactivation), on the other hand, is responsible for their toxicities, which is further exacerbated by their ubiquitous biodistribution after systemic administration. To reduce side effects, the development of GC analogs, which can selectively induce transrepression but not transactivation has been proposed, but with limited success.⁹ Macromolecular prodrug nanomedicine, on the other hand, has been found to significantly improve GC's efficacy and safety.^{10, 11}

Macromolecular prodrug nanomedicine have been used extensively to modulate the PK/BD profiles of drugs in order to improve their therapeutic efficacy and safety.^{12–14} Conjugation

of dexamethasone (Dex) to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer would render Dex nephrotropic in the NZB/W F1 mouse model of lupus nephritis (LN).¹⁵ While evaluating the therapeutic potential of the P-Dex in young lupus-prone NZB/W F1 mice, we reported that it was effective in preventing nephritis development.¹⁶ Later, we found that the monthly treatment of P-Dex would also reverse established albuminuria and improve survival rate in NZB/W F1 mice with established lupus nephritis.¹⁵ In addition to the improved kidney function, P-Dex was found to prevent the development of lupus-associated hypertension, splenomegaly and vasculopathy. Different from the dose-equivalent free Dex treatment, monthly P-Dex treatment does not lead to osteoporosis. Further safety assessment, however, revealed that P-Dex treatment suppressed peripheral white blood cell count and led to adrenal gland atrophy. While the P-Dex design clearly has increased Dex nephrotropicity, it might not have provided enough specificity. Given our previous PK/BD data of P-Dex in inflammatory arthritis and orthopedic implant loosening models,^{17, 18} we speculate that the mononuclear phagocyte system and the blood may have increased exposure to P-Dex, contributing to its residue toxicities.

To address this challenge, we proposed the development of ZSJ-0228 (PEG-Dex), an amphiphilic micelle forming PEG-based polymeric prodrug.^{15, 19} In its structural design, we maintained the same pathology-driven prodrug activation trigger (hydrazone bond, an acid-cleavable linker) as P-Dex, but completely revamped the polymeric carrier design. PEG was selected as the carrier instead of HPMA copolymer because we have found the latter to have a relatively higher phagocytic potential.²⁰ By replacing HPMA copolymer with PEG, we hope to reduce the cell-mediated non-specific sequestration and exposure. Also different from the relatively high molecular weight of P-Dex (35–45 kDa), low molecular weight PEG (1.9 kDa) was chosen as the polymeric carrier to facilitate the prodrug nanomedicine's nephrotropism and kidney clearance.^{17, 18, 20} The Dex dimer-based design renders the prodrug amphiphilic, which would not only increase the Dex loading in the prodrug from ~10 wt % (P-Dex) to 26.4 wt% (ZSJ-0228), but also allow the prodrug to self-assemble into micelles, which would moderate the kidney clearance rate, providing the opportunity for the prodrug to be sequestered by the renal inflammatory cells without being overwhelmed. The validity of this new prodrug design was evaluated in the NZB/W F1 mice with established nephritis.¹⁹ As anticipated, the monthly ZSJ-0228 treatment for two months effectively normalized albuminuria with improved animal survival rate. Most importantly, no typical GC side effects, such as osteoporosis, immunosuppression and adrenal gland atrophy was detected. This improved safety profile of PEG-Dex compared to P-Dex in LN treatment raises a critical question regarding the underlining mechanism of this significant improvement. We posit that the structural difference between P-Dex and ZSJ-0228 may have led to their different PK/BD profiles, and subsequently different safety outcome in the NZB/W F1 mice. To validate this hypothesis, a head-to-head PK/BD comparative study was performed with results presented in this manuscript.

METHODS

Detail information regarding materials and instruments used in this study, the synthesis of ¹²⁵I-labeled HPMA copolymer-dexamethasone conjugate (P-Dex-¹²⁵I) and ¹²⁵I-labeled ZSJ-0228 (PEG-Dex-¹²⁵I) were described in the Supplementary Materials.

Characterization of HPMA copolymer-based Dex prodrug (P-Dex) and ZSJ-0228 (PEG-Dex)

The weight-average molecular weight (M_w), number-average molecular weight (M_n), dispersity (D , M_w/M_n) and Rayleigh Ratio ($R\theta$, dn/dc) of P-Dex were characterized with an ÄKTA pure FPLC system using methods adapted from a protocol provided by Wyatt Technology. To analyze Dex contents in P-Dex and ZSJ-0228 (PEG-Dex), the polymeric prodrugs (4 mg) were dissolved in HCl (0.1 N, 2 mL) and incubated at room temperature overnight for a complete release of Dex. After neutralization with NaOH (0.1 N, 2 mL), the resulting solution was subjected to HPLC analysis, using the following conditions: mobile phase: acetonitrile/water = 2/3; UV detection wavelength: 240 nm; injection volume: 10 μ L; flow rate: 1 mL/min. Mass spectrometry (ESI and MALDI-TOF) was used to characterize the molecular weight of ZSJ-0228 (PEG-Dex).¹⁹

Animal Experiment

A total of 108 NZB/W F1 female mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and utilized in this PK/BD study. All procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center and conducted following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Beginning at 20 weeks of age, weekly monitoring of the mice urine protein level was conducted using Albustix reagent strips (Siemens Healthineers, Erlangen, Germany). Mice with two consecutive albuminuria readings over 2 (100 mg/dL) were considered as having established nephritis.^{16, 19}

The mice were treated with either P-Dex-¹²⁵I (~280 mg/kg) or PEG-Dex-¹²⁵I (~108 mg/kg) by intravenous injection. They contain equivalent dose of Dex at 28 mg/kg as reported previously.^{16, 19} Before injection, the initial dose (ID) of radioactivity was analyzed using a Packard Cobra II Gamma Counter to calculate the radioactivity per milligram prodrug and the radioactivity per mouse received. For each treatment, the treated mice were randomized into 9 groups (6 mice/group) and euthanized at different pre-designed time points post-administration (0.5, 2, 4, 6, 24, 72, 168, 336 and 672 hours). Heart, lung, liver, spleen, kidney, adrenal gland, femur and blood were collected at the time of the sacrifice. For radiation safety consideration, animals were not perfused. The tissues/organs collected were weighed and counted for radioactivity using the gamma counter. The counts from the gamma counter were corrected according to the half-life of ¹²⁵I and the efficiency of the counter. The percentage of initial dose (ID) per gram of tissue (%ID/g) was calculated using the radioactivity of initial dose and radioactivity of each organ/tissue isolated. From the radioactivity of each organ/tissue, the amount of distributed prodrug (P-Dex or ZSJ-0228) was calculated based on the initial radioactivity per milligram prodrug assuming the equal distribution of labeled and un-labeled prodrug.

After gamma counter analyses, the tissues/organs were subjected to free Dex extraction. Approximately 100 mg of tissue isolated (with the exception of adrenal gland, which is typically ~10 mg) was homogenized in 3-volumes of water using Bullet Blender with RINO Bead Lysis Kits (Next Advance, NY, USA). The homogenate was then extracted for free Dex according to a published procedure.²¹ Briefly, 1 mL of organic solvent (ethyl acetate for PEG-Dex sample or methyl *tert*-butyl ether for P-Dex sample) was added to 100 μ L

homogenate. The samples were vortexed for 10 mins and followed by centrifugation at 15,000 g for 10 mins. Supernatant (900 μ L) was collected from each sample and spiked with 10 μ L internal standard (IS, dexamethasone- d_4 , 100 ng/mL in 50% methanol) before evaporated under vacuum with medium heat. The samples were reconstituted in 50% methanol (100 μ L) and vortexed for 3 mins. After centrifugation at 15,000 g for 10 mins, the sample (45 μ L) was analyzed by LC-MS/MS (UPLC-MS/MS, Waters Acquity XevoTQ-XS system, Waters Corp., Milford, Massachusetts, USA). Standard calibrations were prepared using blank tissue homogenate with the same procedure for samples as described above. Since the major content in the tissue homogenate was water, the density of the homogenate was considered as 1 g/mL. With the result from LC-MS/MS in ng/mL, the Dex concentration in the tissue was calculated using the dilution factor and expressed as ng/g.²¹

Pharmacokinetics analysis

Concentrations of the prodrug nanomedicines (P-Dex and ZSJ-0228 which were quantified by radioactivity) and free Dex (quantified by LC-MS/MS) released from the prodrug nanomedicines were calculated for each organ/tissue isolated at every time points. Using the non-compartmental analysis, the averaged concentration of polymeric prodrugs and free Dex versus time profiles were analyzed with WinNonlin Professional software (version 6.3) to produce standard pharmacokinetics (PK) parameters, including area under the drug concentration versus time curve (AUC, expressed as h*ng/g), total clearance (CL, L/h/kg), volume of distribution (V_Z , L/kg) and mean residence time (MRT, h) were calculated. Mean tissue concentrations were calculated and expressed as ng/g tissue.

RESULTS

Different physicochemical properties of the macromolecular prodrug nanomedicines

The P-Dex used in this study was prepared with a M_w (weight average molecular weight) of 41.8 kDa, a M_n (number average molecular weight) of 37.6 kDa and a $\bar{D}$ (dispersity) of 1.1, representing a narrow dispersity. The Dex content in P-Dex was found to be 10.5 ± 0.1 wt%. The Dex content in PEG-Dex was determined to be 25.8 ± 0.7 wt%. According to previous report,¹⁹ the molecular weight of ZSJ-0228 (PEG-Dex) ranges between 2938 Da to 3202 Da and the critical micelle concentration (CMC) value of ZSJ-0228 was determined to be 2.5×10^{-4} M with an average micelle diameter of ~30 nm.

Pharmacokinetics/Biodistribution profiles of ZSJ-0228 (PEG-Dex) and P-Dex in lupus-prone NZB/W F1 mice

The biodistribution of the prodrugs were quantified indirectly by tracking the radioactivity from each organ/tissue assuming the equal distribution of labeled and un-labeled prodrugs. The possibility ^{125}I cleavage from the polymer was discounted due to the absence of radioactivity at the thyroid. The percentage of initial dose (ID) per gram of tissue (%ID/g) vs. time (0.5 h, 2 h, 4 h, 6 h, 24 h, 3 d, 7 d, 14 d, 28 d post-injection) in all collected major organs and tissues were plotted in Figure 1. Overall, the data of ZSJ-0228 (PEG-Dex) exhibits a biphasic pattern post *i.v.* administration, with a rapid distribution from 0.5 to 2 h followed by a relatively slow elimination in most of the organs and tissues examined.

Interestingly, instead of a fast elimination, ZSJ-0228 attained a plateau for up to 24 h in the kidney followed by a rapid elimination.

For P-Dex, the distribution patterns in all the tissues and organs examined are different from ZSJ-0228 (PEG-Dex). In blood, P-Dex showed a steady elimination process. Similar to ZSJ-0228, P-Dex' concentration in kidney was sustained for 24 h, followed by a steady decline, which plateaued from 14 d to 28 d. In the other tissues and organs, P-Dex kept a constant level with very slow elimination. The clearance of P-Dex from liver, spleen and adrenal gland was all very slow. After a quick drop during the initial 0.5 to 2 h post i.v. injection, P-Dex content in liver plateaued at ~2 %ID/g from 2 h to 3 d. After another decline from 3 to 7 d, P-Dex content plateaued at 1.2 % ID/g in liver from 7 to 28 d post i.v. injection. The %ID/g values of P-Dex in spleen and adrenal gland was also sustained at ~1.3 and 2.0, for at least 28 d.

Overall, between the two polymeric prodrugs, ZSJ-0228 (PEG-Dex) showed much quicker clearance than P-Dex in all tissues and organs examined, which is most likely attributed to the distinctively different molecular weights (~3 kDa vs. ~41 kDa) of the two prodrug nanomedicines.

Pharmacokinetics parameters of ZSJ-0228 (PEG-Dex) and P-Dex in lupus-prone mice (NZB/W F1)

Pharmacokinetic (PK) parameters (Tables 1 & 2) of ZSJ-0228 (PEG-Dex) and P-Dex in blood and all major organs/tissues were calculated using non-compartmental analysis. Based on the AUC_{0-28} (total drug exposure from 0.5 h to 28 d) and $AUC_{0-\infty}$ (extrapolated total drug exposure from time zero to infinity) values shown in Table 1, ZSJ-0228 (PEG-Dex) has much higher exposure in kidney and liver than all the other organs/tissues. Also notable is the low $MRT_{0-\infty}$ value (86.79 h) of ZSJ-0228 (PEG-Dex) in kidney, which is significantly lower than the other organs. The $AUC_{\%Extrap}$ values are all lower than 20%, suggesting most of the prodrug nanomedicine have been cleared from these tissues by the last time point (28 d) and that pharmacokinetics parameters estimates are accurate and reliable.²²

The same PK parameters for P-Dex were similarly calculated and shown in Table 2. According to the AUC_{0-28} , the exposure of kidney to P-Dex is higher than spleen, liver, heart, lung and femur but lower than adrenal gland. Although $AUC_{0-28/Kidney/P-Dex}$ is similar to $AUC_{0-28/Liver/P-Dex}$ and $AUC_{0-28/Spleen/P-Dex}$, the $AUC_{\%Extrap}$ of liver and spleen is much higher than kidney, which suggests the P-Dex was retained in liver and spleen longer than that of the kidney.

For PEG-Dex treatment, the $AUC_{0-28/Kidney/ZSJ-0228}$ is similar to $AUC_{0-28/Liver/ZSJ-0228}$, but is ~3 times higher than $AUC_{0-28/Spleen/ZSJ-0228}$ and $AUC_{0-28/Adrenal\ Gland/ZSJ-0228}$. For P-Dex treatment, $AUC_{0-28/Kidney/P-Dex}$ is similar to $AUC_{0-28/Liver/P-Dex}$ and $AUC_{0-28/Spleen/P-Dex}$ but lower than $AUC_{0-28/Adrenal\ Gland/P-Dex}$. Relatively, P-Dex distributed more in spleen and adrenal gland when compared to ZSJ-0228 (PEG-Dex). Almost all organs/tissues examined had much higher exposure (> 10-fold) to P-Dex than ZSJ-0228 (Tables 1&2), which confirms our assessment of the PK/BD profiles shown in

Figure 1. $MRT_{0-\infty}$ are also generally longer for P-Dex than ZSJ-0228, suggesting that it would take much longer for P-Dex to be eliminated from the body than for ZSJ-0228.

Pharmacokinetics/Biodistribution profiles of Dex released from ZSJ-0228 (PEG-Dex) and P-Dex in lupus-prone mice (NZB/W F1)

ZSJ-0228 (PEG-Dex) and P-Dex are both macromolecular prodrug nanomedicine in nature and must be activated to release Dex in order to exert their therapeutic functions. Therefore, to further understand the mechanism of action of these prodrug nanomedicines, all organs/tissues isolated were processed to isolate free Dex for LC-MS/MS analysis. The results were correlated to the PK/BD findings of the prodrugs. The free Dex per gram of tissue (ng/g) vs. time (0.5 h, 2 h, 4 h, 6 h, 24 h, 3 d, 7 d, 14 d, 28 d post-injection) in all collected major organs and tissues are shown in Figure 2, with the exception of adrenal gland due to the very limited amount of tissue (~10 mg) available for extraction. Overall, the amount of Dex released from ZSJ-0228 (PEG-Dex) in all the organs/tissues declined quickly over time post administration except kidney. The concentration of Dex released from ZSJ-0228 (PEG-Dex) in the kidney actually increased from 6 to 24 h post administration, which is likely attributed to the ZSJ-0228 (PEG-Dex) concentration plateau from 2 to 24 h post administration. In most of the tested time points, the average tissue Dex content was lower than 80 ng/g with the exception of the liver and kidney. The average Dex content in liver was found to be ~250–550 ng/g during the first 6 h post ZSJ-0228 injection. The average Dex content in kidney was at ~200 ng/g at 0.5 h and ~100 ng/g at 24h, respectively. The average Dex content in all the other organs/tissues including blood was found to be rather low. For example, the average Dex content in the femur was determined to be ~25 ng/g at 0.5 h post i.v. administration. It was followed by a brief spike to ~36 ng/g at 4 h. At 14 d, average Dex content in the femur was found to be < 1 ng/g. At 28 d, the value further declined to < 0.1 ng/g.

For the Dex released from P-Dex, its content in the blood demonstrated a steady elimination, starting from ~20 ng/g and declined over time. The Dex content was mostly kept unchanged throughout the experiment in the heart (~ 3–13 ng/g), and ranged 4.4–30 ng/g (lung), 12 to 42 ng/g (spleen) and 1–6 ng/g (femur) during the 28 days of the experiment. Similar to what we have observed in the case of ZSJ-0228 (PEG-Dex), the average Dex released from P-Dex showed the highest level in the two clearance organs, the liver and the kidney. The Dex content in liver was found to be ~ 100 ng/g at 0.5 h post P-Dex administration. It gradually increased and peaked at 24 h with a value of ~ 480 ng/g, and followed by a gradual decline to ~ 125 ng/g by 7 d. The Dex content in the kidney followed a similar pattern. It started at ~ 150 ng/g (0.5 h), peaked at ~ 720 ng/g (6 h) and then declined to ~ 15 ng/g (7 d).

Although the Dex content in the kidney of P-Dex treated mice was higher than that of the ZSJ-0228 (PEG-Dex) treated mice, the therapeutic efficacy were found to be similar.^{15, 19} Dex' maximum concentration in the liver of P-Dex treated mice was similar to that of the ZSJ-0228 treated mice, but the timing was at 24 h instead of 2 h as in the case of ZSJ-0228. This delayed appearance of maximum Dex concentration may have resulted from P-Dex' redistribution to and slower clearance from the liver. At the end of the study (28 d post injection), the Dex concentration in liver was at 37.46 ng/g in the P-Dex treated group, while

the Dex level in the ZSJ-0228 group was no longer detectable. A similar trend was also observed in spleen.

Pharmacokinetics parameters of Dex released from ZSJ-0228 (PEG-Dex) and P-Dex in lupus-prone mice (NZB/W F1)

Using WinNonlin, PK parameters for Dex released from ZSJ-0228 (PEG-Dex) and P-Dex were calculated using noncompartmental analysis and shown in Tables 3 & 4. Dex, was released from P-Dex and PEG-Dex, rather than being administered directly. Therefore, in contrast to the total radioactivity data, we only calculated model and administration route-independent PK parameters (AUC, MRT, and C_{max}) for Dex. For Dex released from ZSJ-0228 (PEG-Dex), the two clearance organs (liver and kidney) showed the highest values of Dex AUC_{0-28} in the liver at 10.22 h·μg/g and the kidney at 6.24 h·μg/g. The C_{max} of the Dex released from ZSJ-0228 (PEG-Dex) in these two organs were also found to be the highest with liver at 526.98 ng/g and kidney at 209.12 ng/g.

As shown in Table 4, Dex concentrations in all tissues of P-Dex group were higher than those of the ZSJ-0228 (PEG-Dex) group. Liver, kidney, and spleen had the highest Dex concentrations, with $AUC_{0-28/liver}$ found to be the highest (83.04 h·μg/g), followed by $AUC_{0-28/kidney}$ (27.18 h·μg/g) among all tested organs. The $MRT_{0-\infty}$ of Dex released from P-Dex were found to be ranging from 100 to > 500 h, which were much longer than the corresponding values of Dex released from ZSJ-0228 (PEG-Dex). The C_{max} of the Dex released from P-Dex were found to be higher than 100 ng/g only in liver (484.04 ng/g) and kidney (723.65 ng/g).

The ratio of total P-Dex:PEG-Dex in all tissues were much higher than the ratio of Dex(P-Dex):Dex(ZSJ-0228). For example, $AUC_{0-\infty/Blood/P-Dex}$ is > 10-fold higher than $AUC_{0-\infty/Blood/ZSJ-0228}$, while the $AUC_{0-\infty/Blood/Dex(P-Dex)}$ was just twice the value of $AUC_{0-\infty/Blood/Dex(ZSJ-0228)}$. Another tissue of interest is the femur. While $AUC_{0-\infty/Femur/P-Dex}$ was ~ 100-fold higher than $AUC_{0-\infty/Femur/ZSJ-0228}$, the $AUC_{0-\infty/Femur/Dex(P-Dex)}$ was only 1.7 folds higher than $AUC_{0-\infty/Femur/Dex(ZSJ-0228)}$. These observations suggest that the local prodrug activation mechanisms are critical in defining their overall therapeutic efficacy and safety profiles.

DISCUSSION

Monthly P-Dex was found to effectively ameliorate nephritis in NZB/W F1 lupus-prone mice with 100% survival rate.¹⁵ While P-Dex completely abolished the osteotoxicity associated with dose-equivalent daily Dex, it was still associated with reduced WBC count and adrenal gland atrophy similar to Dex treatment (Table 5). ZSJ-0228 (PEG-Dex) was therefore developed to address the residual GC toxicities of P-Dex.¹⁹ It was not only effective in ameliorating lupus nephritis with 100% survival, but also exhibited significantly improved safety with no osteotoxicity, WBC reduction or adrenal gland atrophy (Table 5). Though a head-to-head comparison study would still be necessary to fully establish ZSJ-0228 (PEG-Dex)'s advantages over P-Dex, these earlier observations^{15,19} prompted us to further inquire of the potential mechanisms underlining its apparent improvement in safety. We posit that the altered polymeric carrier system, especially their different

molecular weights may have led to the different PK/BD parameters, resulting in the different safety profiles.

The PK/BD profiles and the calculated PK parameters of the two macromolecular Dex prodrug nanomedicines and the Dex released, as shown in Results, provide direct evidence that P-Dex and the Dex released from P-Dex were at much higher concentrations in all the organs/tissues than those of ZSJ-0228 (PEG-Dex) in the NZB/W F1 mice. Using a two-compartment model, we have also calculated ZSJ-0228 (PEG-Dex)'s distribution half-life as 0.52 h and its elimination half-life as 11.87 h. Considering the similar therapeutic efficacy of P-Dex and ZSJ-0228 in the amelioration of established lupus nephritis in this model, one would speculate that the P-Dex dose (~280 mg/kg, Dex dose equivalent 28 mg/kg) could be further reduced without compromising its efficacy. For ZSJ-0228 (PEG-Dex), however, the current dosing level (~108 mg/kg, Dex dose equivalent 28 mg/kg) may be adequate due to the absence of GC toxicity.

We recognize that the results from present single dose comparative PK study is preliminary. Dose escalation studies are needed to understand the potential impacts of different dosing levels on the PK parameters of the two macromolecular GC prodrugs. In our initial design of ZSJ-0228 (PEG-Dex), PEG was selected as the carrier polymer in order to reduce potential sequestration by highly phagocytic inflammatory cells in circulation, which may help to limit the systemic distribution of GC. On the other hand, *in vivo* administration of PEGylated therapeutic agents is known to result in generation of anti-PEG antibodies, which could potentially lead to accelerated blood clearance of the drugs.^{23–25} While we speculate that the *in vivo* utility of ZSJ-0228 (PEG-Dex) would not induce anti-PEG antibodies due to the presence of potent immunosuppressive Dex in the prodrug design, and the preliminary result from our recent 5-month repeated dosing treatment study support this notion with sustained therapeutic efficacy (data not shown), a repeated dosing PK study would still be necessary to provide direct evidence. The preexistence of the anti-PEG antibodies in general populations has been reported, though seems to be at low percentage.^{26, 27} Therefore, prescreening for anti-PEG antibodies may be necessary to select proper patient population for the clinical utility of ZSJ-0228 (PEG-Dex).

Due to its higher molecular weight, P-Dex showed much slower clearance and longer resident time in the body than ZSJ-0228 (PEG-Dex), providing ample time for the prodrug to be activated and release Dex. The PK profiles of P-Dex in different organs and tissues, however, do not necessarily mirror the PK profiles of free Dex in the corresponding organs/tissues, suggesting heterogenous prodrug activation mechanisms. At the end of the study (28 d), P-Dex showed much higher accumulation than ZSJ-0228 (PEG-Dex) in liver, spleen, adrenal gland and femur. The slower clearance of P-Dex from spleen may have provided the opportunity for P-Dex' local activation/release of Dex, preventing splenomegaly and vasculopathy as observed in previous study.¹⁵ Given the pivotal roles that the autoreactive B cells in the splenic marginal zone plays in lupus pathogenesis,^{28, 29} the preferential distribution of P-Dex to the spleen may have played a significant role in resolution of lupus symptoms in NZB/W F1 mice. The prolonged resident time of P-Dex in liver and adrenal gland, and the sustained local P-Dex activation and Dex release may have contributed to the reduction of peripheral white blood cells and adrenal gland atrophy.¹⁵ P-Dex' presence in

the femur was also sustained, but at a rather low level (0.2 % ID/g). Furthermore, Dex release from P-Dex in bone was also very low (Figures 1 & 2), which explains the skeletal insensitivity to P-Dex that resulted in the absence of osteopenia.^{15, 16, 19}

Contrary to P-Dex, the clearance of ZSJ-0228 (PEG-Dex) from liver, spleen, adrenal gland and femur was fast (Figure 1). While the amphiphilic PEG-Dex can self-assemble into micelles (~ 30 nm in diameter), which improves the drug loading and water-solubility, the high CMC value (2.5×10^{-4} M) suggests that the micelle is unstable and would quickly disassemble into PEG-Dex monomers once i.v. administered, resulting in fast renal clearance. Combined with the relatively lower levels of free Dex in these organs comparing to P-Dex treated animals and their relatively faster clearance, these data explain the absence of the GC adverse effects (*e.g.*, osteopenia, immunosuppression, adrenal gland atrophy, *etc.*) associated with PEG-Dex treatment.¹⁹ It is interesting to note that ZSJ-0228 did not resolve splenomegaly, which is probably due to its limited distribution to the spleen.¹⁹ Given these findings, it seems that ZSJ-0228 (PEG-Dex) is a “clean-cut” kidney specific GC prodrug nanomedicine that exerts potent, sustained and localized anti-inflammatory and immunosuppressive effect for lupus nephritis. Different from the “carpet bombing” distribution pattern of Dex, P-Dex is a GC prodrug nanomedicine with enhanced nephrotropicity. Its sustained presence and metabolism/activation in the other organs, however, may have been responsible for its residue GC toxicities.

One exception we must consider is the distribution of P-Dex to spleen. As stated above, splenic autoreactive B cells play a very important role in lupus pathogenesis. Previously, we have reported that P-Dex could completely prevent lupus nephritis onset when provided prophylactically. While also very effective in preventing lupus nephritis, preliminary data suggests that ZSJ-0228 (PEG-Dex) could not provide 100 % coverage (data not shown). Given the sustained presence of P-Dex in spleen after *i.v.* administration, we postulate that P-Dex may have been sequestered and activated by the highly phagocytic marginal zone macrophages, providing a sustained splenic Dex level (Figure 2) that lead to autoreactive B cells apoptosis; thus, results in better prevention of lupus nephritis onset when compared to ZSJ-0228 (PEG-Dex). Based upon this assessment, we speculate that the development of an improved version of ZSJ-0228 (PEG-Dex) with dual renal and splenic targeting capacity may be a winning strategy to strike a balance between effective treatment of lupus and complete evasion of GC toxicities.

The outcome from the present PK/BD study also have shed light on the potential underlining mechanism of action of these macromolecular prodrug treatments. P-Dex and ZSJ-0228 were both administered on monthly basis for effective amelioration of nephritis. One may speculate that the sustained therapeutic efficacy of the prodrugs is attributed to their sustained presence and gradual activation within the pathological tissue/organs. The data from the current PK/BD study, however, do not support this notion. As shown in Figures 1& 2 and Tables 1–4, neither P-Dex nor ZSJ-0228 (PEG-Dex) demonstrated long-sustained presence within kidney. The presence of Dex released from the prodrugs within the kidney were only maintained for less than 7 days. As a potential explanation, we speculate that the transient exposure of the nephrotic kidney to effective levels of the polymeric prodrugs may have triggered phenotypic transformation of the relevant renal pathogenic cell populations.

The biological outcome of such phenotypic transformation was sustained for more than a month in the absence of an effective Dex concentration. Preliminary evidence seemed to support such notion. As seen in our previous report, at 4 d post ZSJ-0228 (PEG-Dex) treatment, there was significant reduction of the percentage of myeloid cells (CD11b⁺, CD11c⁺, NK1.1⁺, Ly6G⁺ and F4/80⁺) that were tested positive for the prodrug internalization, when compared to the data from 1 d post treatment.¹⁹ While this snapshot analysis presented preliminary evidence of the renal cell phenotype modification after ZSJ-0228 (PEG-Dex) treatment, it is not sufficient to explain the observed monthlong sustained therapeutic efficacy. Therefore, dynamic analysis of renal cells phenotypic transformation and their biological consequences will have to be thoroughly analyzed in follow-up studies.

In clinical management of rheumatic diseases, several biologics (*e.g.*, Belimumab, Abatacept, Tocilizumab, *etc.*) are maintained on monthly schedule. By conjugating Dex to polymeric carriers, we have dramatically altered the pharmacodynamics of Dex, making them “biologic-like”. The therapeutic efficacies of a single dose P-Dex^{15, 16, 19} and ZSJ-0228¹⁹ can be sustained for at least 1 month, which is much longer than that of the free Dex. Since the monthly dosing schedule for P-Dex and ZSJ-0228 in NZB/W F1 lupus-prone mice was arbitrarily determined according to our previous experience with P-Dex in the treatment of inflammatory arthritis rat model,³⁰ the full extent of their sustained therapeutic efficacy after a single dose is yet to be explored. Clinically, potent and sustained amelioration can also be observed in lupus patients treated with GC pulse therapy.³¹ Adverse effects (*e.g.*, hypokalemia, osteonecrosis of the femur head, *etc.*) associated with such treatment, however, can be prohibitive, which would necessitate close monitoring.³²

Since the inception of nanomedicine, improving the clinical treatment of cancer and more recently infectious diseases have been its main focuses.^{33, 34} For these purposes, pathology-specific and sustained delivery of cytotoxic agents or antimicrobials is essential to achieve disease remission and to avoid the development of drug resistance. As illustrated in the present work, however, when developing nanomedicine for rheumatic disease, the objective to formulate long-sustained formulation may not be necessary and is probably counterproductive due to the potential adverse effects. As the field of nanomedicine shifting its focuses to include inflammatory and autoimmune diseases, this is an important lesson to be gleaned from this study.

In summary, in this head-to-head comparative PK/BD study of P-Dex and ZSJ-0228 (PEG-Dex) and the Dex released from these prodrug nanomedicines, we have quantitatively analyzed and compared the two nanomedicines’ *in vivo* behavior. ZSJ-0228 (PEG-Dex) was found to be a “clean-cut” nephrotropic GC prodrug, while P-Dex has a much higher exposure and broader systemic distribution in addition to nephrotropicity, which explained the superior safety profile of ZSJ-0228 (PEG-Dex) than P-Dex. Splenic targeting has also been identified as a potential beneficial strategy to further strengthen the therapeutic efficacy of ZSJ-0228 (PEG-Dex). As revealed in this study, the “biologic-like” monthlong resolution of lupus nephritis cannot be explained by the transient kidney exposure to high concentrations of these prodrug nanomedicines, which warrant further in-depth investigation of the underlining mechanism of action. Moving forward, the findings from this study will

provide guidance to further optimize macromolecular dexamethasone prodrug nanomedicine design for better and safer clinical management of lupus nephritis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Though similar in treatment efficacy, P-Dex and PEG-Dex showed dramatically different PK/BD profiles on NZB/W F1 lupus-prone mice.
- The systemic organ/tissue exposures to P-Dex and the Dex released from P-Dex were found to be significantly higher than those of PEG-Dex.
- Though the therapeutic effects of P-Dex and PEG-Dex lasted for more than a month, their high renal concentrations were sustained for only 24 hours.

Figure 1.
Pharmacokinetics/biodistribution (PK/BD) profiles of P-Dex and ZSJ-0228 (PEG-Dex) in NZB/W F1 lupus-prone mice over 28 days post single i.v. administration. (n = 5~6).

Figure 2. Pharmacokinetics/biodistribution profiles of free Dex released from P-Dex and ZSJ-0228 (PEG-Dex) in NZB/W F1 lupus-prone mice over 28 days post i.v. administration. (n = 5~6). The data points without lower error bar represent samples with free dexamethasone concentrations below detection limit of LC-MS/MS.

Table 1.

The pharmacokinetic (PK) parameters of ZSJ-0228 (PEG-Dex) after systemic administration from non-compartmental analysis.

Pharmacokinetics parameters	Blood	Heart	Liver	Spleen	Lung	Kidney	Adrenal Gland	Femur
$t_{1/2}$ (h)	149.71	329.71	248.96	331.33	271.31	201.18	277.46	318.76
AUC_{0-28} (h·mg/g)	2.04	0.99	6.12	2.20	1.57	6.87	2.32	0.50
$AUC_{0-\infty}$ (h·mg/g)	2.05	1.11	6.78	2.71	1.68	7.04	2.69	0.58
$AUC\%_{\text{Extrap}}$ (%)	0.5	11.1	9.8	18.7	6.6	2.4	13.6	12.6
V_z (L/kg)	11.39	46.29	5.72	19.06	25.19	4.45	16.07	86.22
CL (L/h/kg)	0.05	0.10	0.02	0.04	0.06	0.02	0.04	0.19
$MRT_{0-\infty}$ (h)	36.65	223.11	235.12	361.93	151.04	86.79	301.91	258.96

$t_{1/2}$, the half-life associated with the elimination phase; CL, total body clearance; V_z , volume of distribution during terminal phase; AUC, area under the concentration of Dex-containing prodrugs vs time curve; MRT, mean residence time.

Table 2.

The pharmacokinetic (PK) parameters of P-Dex after systemic administration from non-compartmental analysis.

Pharmacokinetics parameters	Blood	Heart	Liver	Spleen	Lung	Kidney	Adrenal Gland	Femur
$t_{1/2}$ (h)	71.97	750.50 *	1,982.27 *	3,350.64 *	379.29 *	71.57	889.24 *	1,093.10 *
AUC ₀₋₂₈ (h·mg/g)	28.57	33.72	107.70	99.74	44.41	111.12	167.92	16.31
AUC _{0-∞} (h·mg/g)	28.89	69.16 *	499.39 *	780.56 *	59.91 *	115.91	411.67 *	43.77 *
AUC%Extrap (%)	1.13	51.25	78.43	87.22	25.88	4.14	59.21	62.74
V _z (L/kg)	1.01	4.38 *	1.60 *	1.73 *	2.56 *	0.25	0.87 *	10.09 *
CL (mL/h/kg)	9.69	4.05 *	0.56 *	0.36 *	4.67 *	2.42	0.68 *	6.40
MRT _{0-∞} (h)	76.50	1,032.50	2,837.43	4,844.96	494.68	175.49	1,289.95	1,521.26

$t_{1/2}$, the half-life associated with the elimination phase; CL, total body clearance; V_z, volume of distribution during terminal phase; AUC, area under a concentration of analyte vs. time curve indicating total drug exposure; MRT_{0-∞}, mean residence time indicating mean time of each drug molecule stays in the body.

* indicates the calculated parameter may not be accurate as the AUC%Extrap is > 20%, suggesting a significant portion of the drug is still in the body.

Table 3.

The pharmacokinetic (PK) parameters of Dex from ZSJ-0228 (PEG-Dex) after systemic administration.

Pharmacokinetics parameters	Blood	Heart	Liver	Spleen	Lung	Kidney	Femur
AUC ₀₋₂₈ (h·µg/g)	0.64	0.73	10.22	3.56	2.24	6.24	1.73
AUC _{0-∞} (h·µg/g)	0.64	1.04	10.22	3.73	2.25	6.31	1.75
C _{max} (ng/g)	80.69	13.97	526.98	31.79	61.04	209.12	36.03
MRT _{0-∞} (h)	63.16	454.98	23.93	259.12	80.26	49.21	117.92

AUC, area under a concentration of analyte vs. time curve indicating total drug exposure; MRT, mean residence time indicating mean time of each drug molecule stays in the body.

Table 4.

The pharmacokinetic (PK) parameters of Dex from P-Dex after systemic administration.

Pharmacokinetics parameters	Blood	Heart	Liver	Spleen	Lung	Kidney	Femur
AUC ₀₋₂₈ (h·µg/g)	1.22	4.29	83.04	14.22	97.64	27.18	2.34
AUC _{0-∞} (h·µg/g)	1.28	5.99	99.00	19.58	11.96	28.12	31.14
C _{max} (ng/g)	18.32	13.25	484.04	41.91	29.39	723.65	5.61
MRT _{0-∞} (h)	251.26	558.92	329.67	508.80	400.48	105.48	492.46

AUC, area under a concentration of analyte vs time curve; MRT, mean residence time.

Table 5.

Comparison of safety parameters of P-Dex and ZSJ-0228 (PEG-Dex) *

Parameters	P-Dex study ¹⁵			ZSJ-0228 (PEG-Dex) study ¹⁹		
	P-Dex	Dex	Saline	P-Dex	Dex	Saline
BMD (mg/cm ³)	207.6±31.6 [↑]	166.1±26.7 [⊥]	203.3±34.2	209.1±24.3 ^{↑;⊥}	155.6±30.0	155.1 ±70.7
WBC (count/μL)	1537±751 [⊥]	2192±698 [⊥]	4202±899	3743±393 [↑]	2410±1043	3321±891
ADW (mg)	8.4±2.5 [#]	8.2±1.4 [⊥]	11.4±3.9	9.6±2.2 [↑]	6.4±0.9	8.0±2.2

* Data were extracted from references.^{15, 19} BMD: bone mineral density; WBC: white blood cell; ADW: adrenal gland weight.

[↑] Significantly different from Dex group;

[⊥] Significantly different from Saline group.

[#] P-Dex vs. Saline, $P = 0.0825$. Tukey's multiple comparisons test.