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Hepatic Immunosuppressive Effects of Systemically-Administered Novel Dextran-Methylprednisolone Prodrugs with Peptide Linkers in Rats

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Abstract

The hepatic immunosuppressive activities of two novel dextran prodrugs of methylprednisolone (MP) containing one (DMP1) or five (DMP5) amino acids as linkers were studied in rats. At various times (0–2 weeks) after intravenous administration of single 5-mg/kg (MP equivalent) doses of each prodrug or MP succinate (MPS), livers were isolated and immunologically stimulated *ex vivo* with lipopolysaccharide. The concentrations of tumor necrosis factor (TNF)-α in the outlet perfusate were then quantitated to assess immune response. Additionally, the concentrations of DMP1, DMP5, and/or MP were measured in the liver. MPS, DMP5, or DMP1 injections caused a maximum of 48.9%, 63.5%, or 85.7% decrease in the TNF-α secretion into the perfusate, with the time above the 50% inhibitory effect being <5, <24, or 120 h, respectively. Additionally, the area under the effect-time curve for DMP1 was 11- or 4-fold higher than that after the administration of MPS or DMP5, respectively. Relatively high concentrations of DMP1 were present in the liver even at the last sampling time of two weeks. These data suggest that a single intravenous dose of DMP1 produces an intense and sustained immunosuppression in the liver for a relatively long time, which may be useful in liver transplantation.

Keywords

dextran prodrugs; macromolecular prodrugs; peptide linkers; methylprednisolone; pharmacokinetics; liver perfusion; hepatic immunosuppression; hepatic delivery

INTRODUCTION

The corticosteroid methylprednisolone (MP) has been used as an immunosuppressive agent in liver transplantation for induction and early maintenance of the immunosuppression and also for the treatment of acute rejection of the graft.[1–5] For the induction purposes, MP is usually administered intravenously immediately after the graft reperfusion at a high dose of 500–1000 mg.[3,5] Additionally, MP at high intravenous bolus doses of 1000 mg per day, administered over several days, is the first-line therapy for the treatment of acute liver graft rejection.[1,2,4] Despite a high degree of effectiveness, the large intravenous doses of MP are associated with substantial toxicities, sometimes with fatal consequences.[6–11] Therefore, strategies to reduce the toxicity of large dose MP therapy are highly desirable.

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It has been proposed that selective immunosuppression in the graft might result in lower toxicity that is related to excessive systemic immunosuppression, such as life-threatening infections.[12] Additionally, graft-selective immunosuppression would allow administration of lower doses of the drug, further reducing the possibility of the overall side effects of the drug not related to immunosuppression, such as cardiovascular[13,14] and central nervous system[15–17] toxicities observed with the high dose MP. So far, different approaches have been utilized to achieve graft-selective immunosuppression. These approaches mainly include injection of the immunosuppressive drug directly into the vessels that supply the blood to the transplanted organ[18–20] or formulation approaches to target the graft after systemic administration.[21,22] Both of these approaches have been effective in various experimental transplantation models.

We have used a macromolecular prodrug approach, based on neutral dextran carriers, for the selective delivery of MP to the liver for the purpose of induction of immunosuppression and/or treatment of acute rejection in liver transplantation. First, a conjugate linking MP to a 70 kDa dextran using a succinate linker was synthesized, and its pharmacokinetics[23] and pharmacodynamics[24–26] were characterized in rats. After the systemic administration, the conjugate selectively accumulated in the rat liver[23] and was much more effective than the parent drug in suppression of immune events in the liver.[24,26] However, the release of MP from the conjugate was slow and incomplete.[24,25]

To control the *in vivo* rate of release of MP from the prodrug, second-generation dextran conjugates were synthesized using peptides of different lengths as linkers.[27] *In vitro* release studies showed that the rate of release of MP from the prodrugs was positively related to the length of the peptide linker when Gly and/or methyl Gly (mGly) were used as amino acids.[27] Further *in vivo* studies[28] in rats using prodrugs containing one (DMP1) or five (DMP5) amino acids as linkers showed a significant effect of the linker length on the pharmacokinetics and tissue disposition of the prodrugs and the released MP. Whereas the extent of accumulation of DMP1 in the liver, spleen, and kidneys was much higher than that of DMP5, the rate of release of MP after DMP5 injection was faster than that of DMP1. However, whether and how these pharmacokinetic differences affect the immunosuppressive effects of the prodrug in the liver are not known at this time. Therefore, the purpose of the current study was to determine the hepatic immunosuppressive effects of DMP1 and DMP5, in comparison with that of an equivalent dose of the parent drug MP, after the systemic administration of the prodrugs to rats. Based on the reported disposition studies,[28] our hypothesis was that whereas both prodrugs are more effective than the parent drug, the DMP1 prodrug would produce the most intense and sustained immunosuppression in the liver.

MATERIALS AND METHODS

Chemicals

Dextran (average M_w: ~23500, degree of polydispersity: 2.3) was obtained from Dextran Products, Ltd. (Scarborough, Ontario, Canada). *Escherichia coli* (Serotype 0111:B4) lipopolysaccharide (LPS), sodium taurocholate, 6α-methylprednisolone succinate (MPS), and internal standard (triamcinolone acetonide) were purchased from Sigma Chemical Company (St. Louis, MO). Rat tumor necrosis factor (TNF)-α ELISA kit (ER3TNFA) was obtained from Thermo Scientific (Rockford, IL). Kits for determination of transaminases were purchased from Teco Diagnostics (Anaheim, CA). All other chemicals were of analytical grade and obtained from commercial sources.

Dextran-methylprednisolone conjugates with methyl Gly (mGly) (DMP1) or mGly-Gly-Gly-Gly-Gly (DMP5), as the linkers between the polymer and MP, were synthesized and

characterized as previously reported by us.[27] The degrees of substitution (w/w) of the conjugates were 10.8% and 7.6% for DMP1 and DMP5, respectively, with purities of > 95%.

Experimental Design

All procedures involving animals used in this study were consistent with the guidelines set by the National Institutes of Health (NIH publication # 85-23 revised 1985) and approved by our Institutional Animal Care and Use Committee.

Adult, male Sprague-Dawley rats were obtained from Charles River laboratory (Wilmington, MA) and housed in a 12-h light-dark cycle and temperature-controlled facility with free access to rat chow and drinking water at all times. A total of 59 animals were used for the entire study. Seventeen groups of animals with 3 animals per group were used to study the time courses of the effects of MPS (at 5, 12, 24, and 48 h), DMP1 (at 5, 12, and 24 h and 2, 3, 5, 8, and 14 days), and DMP5 (at 5, 12, and 24 h and 2 and 5 days) on the LPS-stimulated release of TNF-α. A single dose of MPS, DMP1, or DMP5, equivalent to 5 mg/kg MP, was administered intravenously via the penile vein under isoflurane anesthesia. At various times after each drug administration described above, rats were anesthetized, and their livers were isolated and stimulated *ex vivo* with LPS using an isolated perfused rat liver (IPRL) model. Additionally, a negative ($n = 3$) and a positive ($n = 5$) control group were included in the study. The control groups did not receive any drug pretreatment before the liver isolation. However, the livers of the positive control groups were stimulated *ex vivo* with LPS, whereas the livers of the negative control group were not subjected to the LPS stimulation.

Ex Vivo Perfusion and LPS Stimulation of the Isolated Livers

An IPRL model was used to test the hepatic immunosuppressive activity of the systemically-administered MPS, DMP1, or DMP5. This *ex vivo* model is based on the stimulation of livers with LPS and the measurement of TNF-α released in the outlet perfusate, as described in detail before.[29] Briefly, after cannulation and isolation, the livers were transferred to a temperature (37°C)-controlled chamber and perfused in a single-pass manner at 30 mL/min (3–4 ml/g wet liver weight) for 120 min. The perfusate consisted of Krebs-Henseleit bicarbonate buffer (pH 7.4), supplemented with 1.2 g/L glucose and 4.75 mg/L sodium taurocholate, and was oxygenated with a 95:5 oxygen: carbon dioxide mixture.

The livers were mounted onto the IPRL system and allowed to stabilize for ~15 min. Except for the negative control group, the livers were immunologically stimulated with 1 mL of a 300 μg/mL solution of LPS, which was infused at a rate of 50 μL/min for 20 min.[29]

Liver viability was evaluated as described before[30–32] via macroscopic examination, relatively stable bile flow rates during the entire period of perfusion, low and stable inlet pressure, low concentrations of aspartate (AST) and alanine (ALT) aminotransferases in the perfusate at the beginning and end of the perfusion, and wet liver weights of <4% of total body weight at the end of perfusion.

Sample Collection

Outlet perfusate samples (~1 mL) were collected at 0, 15, 30, 45, 60, 80, 100, and 120 min for the measurement of TNF-α. Additional samples were collected at the start and end of perfusion to determine the liver injury markers. Bile samples were collected in pre-weighed tubes at 30 min intervals, and bile flow rates were determined gravimetrically. At the completion of the perfusion, the livers were removed from the perfusion system, cleaned

from the muscle and extraneous tissues, blotted dry, and weighed. The perfusate, bile, and liver samples were stored at −80°C until further analysis.

Sample Analysis

The concentrations of TNF-α in the outlet perfusate were quantified by ELISA according to manufacturer's directions. The assay, which uses a 50-μL sample, has a sensitivity of < 15 pg/mL with an inter- and intra-assay coefficient of variation of < 10%. The AST and ALT levels in the perfusate were determined based on a colorimetric assay using commercial kits.

The concentrations of MP, DMP1, and DMP5 in the liver were determined according to the procedures reported by us before. Briefly, livers were homogenized in 2% acetic acid (1:3), and the homogenate was used in the assay. For MP determination, a reverse phase HPLC method was employed[33] with a modified extraction procedure.[28] Briefly, 600 μL of cold acetonitrile containing 5 μg/mL triamcinolone acetonide (internal standard) was added to 240 μL of the liver homogenate, and the samples were vortex mixed for 30 sec and centrifuged at 14000 rpm for 5 min. The supernatant was transferred to a centrifuge tube and dried under a nitrogen stream. The dried residue was dissolved in 120 μL of methanol followed by the addition of 120 μL of 10 mM sodium acetate buffer pH 4.5. A 100 μL aliquot of the resultant solution was injected into the column. The samples were analyzed at ambient temperature utilizing a 15 cm × 4.1 mm polystyrene-divinylbenzene copolymer (5 μm) column (PRP-1; Hamilton, Reno, Nevada), equipped with a 5-cm guard column packed with pellicular C_{18} material. The mobile phase consisted of 100 mM acetate buffer (pH 5.7): acetonitrile (77:23) pumped at 1.5 mL/min. The drug and internal standard were detected at 250 nm.

The concentrations of DMP1 and DMP5 in the liver homogenates were determined using a size-exclusion assay reported by us[28,34] with some modifications. To 100 μL of the liver homogenate were added 50 μL of acetonitrile, 50 μL of methanol, and 20 μL of 70% perchloric acid, and the samples were vortexed for 30 seconds and centrifuged at 14000 rpm for 5 min. A 170 μL aliquot of the supernatant was then transferred to a new tube and 1 mL of cold acetonitrile was added to precipitate the dextran conjugates. After vortex-mixing and centrifugation, the supernatant was decanted, and the tube was dried for 3 min by inversion on a paper towel. The residue was dissolved in 200 μL of mobile phase, and 100 μL was injected into a 25 cm × 4.6 mm Nucleosil 100–7 OH column (Macherey-Nagel, Bethlehem, PA). The analytes were eluted with a mobile phase of 10 mM KPO_4 (pH 6.0): acetonitrile (55:45) with a flow rate of 1 mL/min.

Data Analysis

The area under the TNF-α concentration (outlet perfusate)-time curve (AUC) was calculated using linear trapezoidal rule. The inhibitory effects of MP, DMP1, or DMP5 pretreatment on the TNF-α secretion into the outlet perfusate were calculated as the percent inhibition of TNF-α AUC using the following equation:[24]

$$\%\text{Inhibition} = \frac{AUC_{Positive\ Control} - AUC_{\text{Pretreatment}}}{AUC_{Positive\ Control}} \times 100 \quad (1)$$

where $AUC_{Positive\ Control}$ and $AUC_{Pretreatment}$ refer to the TNF-α AUC in the positive control group and that after the pretreatment of rats with MPS, DMP1 or DMP5, respectively. The %inhibition of TNF-α was plotted against the time after the drug administration, and the population mean and variance of the area under the effect (%inhibition of TNF AUC)-time curve (AUEC) were estimated using sparse sampling

model in Phoenix® WinNonlin® software (Pharsight, Sunnyvale, California). The mean and variances of the AUC of MP, DMP1, or DMP5 in the liver during the sampling time (AUC_{last}) or from time zero to infinity (AUC_{∞}) were also estimated by linear trapezoidal rule for the sparse data using the same software. The concentration-time courses of DMP1 and DMP5 in the liver were fitted to the following biexponential equation:

$$C=C_1e^{-k_1t}+C_2e^{-k_2t} \quad (2)$$

where C_1 and C_2 are the concentration coefficients and k_1 and k_2 are the apparent first order rate constants for the fast and slow decline phases, respectively. The population mean and variance of the four parameters in the equation were estimated using the Phoenix® NLME® software.

Statistical Analysis

The effect of drug pretreatment on the TNF-α AUC in the outlet perfusate for different time points was determined using one-way ANOVA followed by Dunnett's post-hoc analysis, comparing means for each time point after drug treatment with the positive control mean. The effects of treatments on the average bile flow rates were determined by one-way ANOVA, followed by Tukey's Multiple Comparison test. The differences among MPS, DMP1, and/or DMP5 in terms of their AUECs or liver AUCs, C_1, C_2, k_1, and k_2 were analyzed using a two-sided Z-test after Bonferroni adjustment for the appropriate number of comparisons, as described in detail before.[23] All comparisons were performed at a significance level (α) of 0.05 after Bonferroni adjustment, if applicable. The data are presented as mean ± SD for parameters that could be estimated for individual animals (e.g., TNF-α AUC, bile flow rate, maximum effect, and maximum liver concentration) and as mean ± SE for population parameters (e.g., AUEC and liver AUC and bi-exponential parameters of prodrugs).

RESULTS

The bile flow rates for all the treatment groups were relatively high (Fig. 1) and stable during the four collection intervals over the 2 h of perfusion (individual interval data not shown). Neither LPS nor drug pretreatments significantly ($p > 0.05$) affected the average bile flow rates (Fig. 1).

The outlet perfusate concentration-time profiles of TNF-α in the negative and positive control groups are shown in Fig. 2. Also depicted in the figure are the TNF-α profiles for MPS at 5 h, DMP1 at 24 h, and DMP5 at 12 h after the drug treatments, representing the peak effects of these treatments. For brevity, the TNF-α profiles at the other times after the drug treatments are not shown. The data show very low concentrations of TNF-α in the perfusate of the negative control livers. However, positive control livers show a delayed, but steep increase in the TNF-α concentrations in the perfusate, starting at 30 min after the stimulation with LPS (Fig. 2). All three drug treatments substantially reduced the LPS-stimulated TNF-α release in the outlet perfusate, with the highest magnitude of the effect seen with DMP1 (at 24 h), followed by DMP5 (at 12 h) and MPS (at 5 h) (Fig. 2). In fact, the TNF-α profile at 24 h after the administration of DMP1 was almost indistinguishable from the profile in the negative control group (Fig. 2).

The extent of suppression of TNF-α release after various pretreatments at each time point may be quantified by calculating the AUC of TNF-α concentration-time curves, which are presented in Table 1. The LPS stimulation of the livers without pretreatment (time zero in Table 1) resulted in a significant increase in the TNF-α AUC, as compared with the AUC in the negative control group, which was close to zero. Pretreatment with MPS showed a

significant reduction ($p < 0.05$) in the TNF-α AUC at 5 h after the drug treatment. However, the AUCs at the later time points after the MPS injections were not significantly different from the AUC at time zero (positive control). In contrast to MPS, DMP1 injections resulted in significant ($p < 0.01$) reductions in the TNF-α AUC lasting for up to 5 days after the drug injection. On the other hand, the reductions in the TNF-α AUC after DMP5 injection, although lasting longer than those after the MPS injection, were not significant beyond 12 h following the conjugate injection (Table 1).

The effects of various drug pretreatments, expressed as % inhibition of TNF-α AUC, at different times after the drug administration are presented in Fig. 3. Additionally, the associated pharmacodynamic parameters are presented in Table 2. The maximum effect of MPS treatment was 49% reduction in the TNF-α AUC, which occurred at the first sampling time of 5 h. Thereafter, the effect decreased rapidly with less than 15% inhibitory effect remaining at the last sampling point of 48 h (Fig. 3). Pretreatment with DMP5 resulted in a maximum inhibitory effect of 64% observed at 12 h after the drug injection, which was not significantly higher than the maximum effect after the MPS injection (Table 2). However, the immunosuppressive effect of DMP5 lasted longer than that of MPS, falling below 15% at 5 days after the injection (Fig. 3). Among the 3 drug pretreatments, DMP1 showed the highest inhibitory effect (86%), which was observed at 24 h after the drug injection (Fig. 3 and Table 2). Additionally, the effect of DMP1 was much more sustained than that of either MPS or DMP5, with the last sampling time of 14 days showing a 26% inhibitory effect (Fig. 3). In terms of the overall effect, the area under the effect (%inhibition of TNF AUC)-time curve (AUEC) values, calculated for the duration of sampling, were 2.5 and 11 fold higher for DMP5 and DMP1, respectively, when compared with the parent drug MPS (Table 2). In addition, the time above 50% inhibition for DMP1 (120 h) was substantially longer than that for the MPS and DMP5 pretreatments (Table 2).

The liver concentration-time curves of DMP1 and DMP5 and the released active molecule, MP, after a single 5-mg/kg dose (MP equivalent) are shown in Fig. 4. Additionally, the associated non-compartmental pharmacokinetic parameters are listed in Table 3. Although after the MPS injection the liver concentrations of MP were not detectable in any of the samples, relatively high concentrations of DMP1 and DMP5 were quantifiable in all the measured samples (Fig. 4). Additionally, the released MP was quantifiable for up to 12 and 48 h after the DMP5 and DMP1 injections, respectively (Fig. 4). For both DMP1 and DMP5, the highest liver concentration was observed at the first sampling time of 5 h. However, the maximum concentration for DMP1 (37.9 μg/g) was almost 8 times higher than that for DMP5 (4.82 μg/g) (Table 3). The higher liver concentration of DMP1, compared with DMP5, was reflected in 9 and 5 fold higher AUC_{last} and AUC_{∞}, respectively, for this conjugate (Table 3). In agreement with the data for the prodrugs, the C_{max} and AUC_{last} of the regenerated MP after DMP1 treatment were 3.6 and 17 fold higher than those after the DMP5 treatment (Table 3).

The results of the biexponential fit of the liver concentration-time data for DMP1 and DMP5 are presented in Table 4. As expected from Fig. 4, the concentration coefficients (C_1 and C_2) for DMP1 were significantly higher than the corresponding values for DMP5. As for the rate constants, although the terminal rate constants (k_2) were similar for both prodrugs, the rate constant associated with the fast decline in the liver concentrations (k_1) for DMP5 was 3.7 fold higher than that for DMP1 (Table 4).

The plots of % inhibition of TNF-α AUC versus DMP1 and DMP5 concentrations in the liver are shown in Fig. 5. Both DMP1 and DMP5 showed indications of counter-clockwise hysteresis loops, suggesting that the effect is delayed relative to the appearance of the prodrug in the liver. The plots also suggest that at similar liver concentrations, DMP5 and

DMP1 produce similar effects as the effect-concentration data points for DMP5 are almost superimposable on the data points related to the lower liver concentrations of DMP1 (Fig. 5).

DISCUSSION

In the current study, we compared the hepatic immunosuppressive activities of MP and its two recently-developed novel prodrugs after their systemic administration. Although all the pretreatments used here (MPS, DMP1, and DMP5) showed immunosuppressive activities in the liver, the intensity and duration of the effect was formulation dependent. The rank order for the intensity of the maximum effect and the duration of effect was DMP1 > DMP5 > MPS (Figs. 2 and 3 and Table 2), which was in apparent agreement with the extent of the exposure of the liver to the prodrugs and/or the active moiety (i.e., MP) (Fig. 4). After administration of MPS, we could not detect MP in the liver samples, which were taken ≥ 5 h after the drug injection. This is consistent with our previous report[28] showing that the liver concentrations of MP reach their maximum shortly after the administration of the parent drug and are not detectable beyond 2 h after a single 5-mg/kg dose of the drug. In contrast to the MPS injection, we were able to quantify MP regenerated from DMP5 and DMP1 for 12 and 48 h, respectively, following equivalent doses (Fig. 4). Overall, these data suggest that DMP1 exhibits optimal pharmacokinetic and pharmacodynamic characteristics for situations requiring intense and sustained immunosuppression in the liver.

The liver disposition data in Fig. 4 and Table 4 reveal some major differences between the two conjugates in their hepatic disposition. First, the lower concentration coefficients (C_1 and C_2) for DMP5 indicate a lower extent of liver accumulation for this conjugate relative to that for DMP1. Additionally, the observation that the concentrations of both DMP1 and DMP5 in the liver decline in a biexponential manner is suggestive of the presence of two processes contributing to the decline in the liver concentrations of the conjugates. Because dextrans by themselves do not have a chromophore in their structure, the decline in the liver concentrations of the conjugate is most likely due to the release of MP from the conjugate. Therefore, the rate constants k_1 and k_2 are most likely representative of a fast and a slow release process, respectively. The reasons for the two release processes (Table 4) are not clear based on our current data. However, one may speculate that the conjugation of MP to different hydroxyl groups of the glucose monomer may result in different types of conjugates with different degrees of steric hindrance for access by peptidases. Nevertheless, these data (Table 4) suggest that whereas the slower release process, represented by k_2, is similar for both conjugates, the fast release process, represented by k_1, is ~4 fold higher for DMP5, compared with DMP1.

Although the pharmacokinetics and pharmacodynamics of a first-generation dextran-MP conjugate with a succinate linker (DMP-Succinate) have been investigated relatively extensively, this study is the first to investigate the pharmacodynamics of the newly-developed dextran-peptide-MP conjugates. There are only two publications[27,28] available related to these novel conjugates. The first publication[27] described the synthesis and *in vitro* release characteristics of a series of dextran-MP conjugates with Gly peptide linkers of different lengths. The *in vitro* release data using peptidases or rat liver lysosomal fractions showed a linear positive relationship between the rate of MP release from the conjugate and the length of the peptide linker. Our *in vivo* data showing ~4 fold higher k_1 for DMP5, compared with DMP1 (Table 4), appear to be in agreement with these *in vitro* data.[27]

The second publication dealing with the novel DMP conjugates investigated the relatively short-term (12–24 h) plasma and tissue pharmacokinetics of DMP1 and DMP5.[28] Whereas the conjugates were not detectable in lungs, heart, and brain, relatively high concentrations

of the conjugates were found in the liver, spleen, and kidney, suggesting selective accumulation of the conjugates in the latter tissues. Similar to our current observation (Fig. 4), the liver concentrations of DMP1 were substantially higher than those of DMP5 in the previous report.[28] However, in contrast to our current data (Fig. 4), the conjugates showed an apparent monoexponential decline in their liver concentration-time courses in the published study. This apparent discrepancy is most likely due to the shorter sampling times (12 and 24 h for DMP5 and DMP1, respectively) in the published study.[28] In fact, if one considers only the first 12 (DMP5) or 24 (DMP1) h data in the current study (Figure 4), the multiexponential nature of the data would disappear. The longer sampling times, resulting in the detection of a second slower phase in the current study, is also responsible for the higher liver AUC values reported in Table 3, compared with the data in the previous study.[28] Overall, the liver disposition data reported here (Fig. 4 and Table 4) agree with and extend our previous knowledge[28] about the fate of these conjugates in the liver.

Although this is the first report on the pharmacologic activity of the novel conjugates of MP with amino acid/peptide linkers, the pharmacodynamics of a different dextran prodrug of MP with a succinate linker (DMP-Succinate) have already been reported.[24] The data presented in this manuscript suggest that not only is DMP1 superior to DMP5 (Fig. 3 and Tables 1 and 2), it has also more suitable pharmacodynamic characteristics than the conjugate with the succinate linker (DMP-Succinate). Whereas the peak effect time (24 h for both) and the maximum inhibitory effect (86% for DMP1 and 80% for DMP-Succinate) are similar for the same dose of the prodrugs, the duration of the effects of DMP1 is much longer than that of DMP-Succinate. This conclusion is based on a significant inhibition of TNF-α AUC by 2 days for DMP-Succinate,[24] as compared with a 5-day inhibition for DMP1 (Table 1). Additionally, the AUEC of DMP1 determined in the current study (638 %inhibition.day) is >four fold higher than that of DMP-Succinate (153 %inhibition.day).[24] These data indicate that at equivalent MP doses, DMP1 produces a more sustained effect than the first generation DMP-Succinate prodrug.

The significant differences between DMP1 and DMP-Succinate in terms of their length of the effect could be attributed to the differences between the prodrugs in terms of the extent of their liver accumulation and/or rate of MP regeneration. However, our previous study[24] showed that the concentrations of DMP-Succinate in the liver during the 72 h of measurement were similar to or higher than those observed here for DMP1 (Fig. 4). Therefore, the longer effect of DMP1, observed in the present study, compared with that of DMP-Succinate, reported before, is most likely due to the differences between the prodrugs in terms of release of MP.

The effect-liver concentration plots for DMP1 and DMP5 suggest the presence of a counter-clockwise hysteresis (Fig. 5). This is in agreement with our previous report for DMP-Succinate, which also showed a counter-clockwise hysteresis in its effect.[24] Theoretically, a counter-clockwise hysteresis for a prodrug may be due to a delay in the regeneration of the active drug. However, the counter-clockwise hysteresis was also observed when the effects was plotted against the regenerated MP after DMP-Succinate, administration.[24] These observations are in agreement with previous studies[29,35] reporting a several-hour delay between the injection of MP and inhibition of LPS-stimulated TNF-α release.

The exact mechanisms of the inhibitory effects of glucocorticoids on the LPS-stimulated TNF-α release are not fully understood. It has been reported[36,37] that glucocorticoids, such as MP, act at several points in stimulated macrophages or monocytes to prevent the release of TNF-α by inhibiting TNF-α gene transcription and/or translation. *In vitro* data has shown that LPS stimulation transiently increases the expression of TNF-α gene.[37] Although glucocorticoids antagonize this enhanced expression, they do not affect the TNF-α gene

expression in the absence of LPS stimulation. Additionally, the data in murine cells suggest that the glucocorticoids inhibit the enhanced translation of TNF-α mRNA in stimulated monocytes.[38] Overall, the available data indicate that the inhibitory effects of MP on TNF-α release are indirectly related to the liver concentrations of MP. Therefore, the apparent counter-clockwise hysteresis observed in the DMP1 and DMP5 effects (Fig. 5) are due most likely to the delay in the effects of MP and not the release of MP from the prodrugs. Indeed, in agreement with our data, Waage[35] reported that the maximum inhibitory effects of dexamethasone on TNF-α release in rats occurred when dexamethasone was administered 5 h before the injection of LPS.

In addition to the use of dextrans, other approaches have also been reported for targeted delivery of MP[39] or other corticosteroids[40] to the liver or other organs of reticuloendothelial system. For example, Mishina *et al.*[39] developed a liposomal formulation of MP and showed its superior immunosuppressive activities, over the parent drug, in a heart transplantation model.[41] However, the immunosuppressive activity of the formulation in the liver was not reported. Additionally, Melgert *et al.*[40] synthesized an albumin conjugate of dexamethasone, which was selectively distributed into the non-parenchymal cells of the liver. However, the overall accumulation of the conjugate in the liver was not substantially different from that after the administration of the parent drug. Nevertheless, the substantial and sustained accumulation and effects of DMP1 in the liver over a period of two weeks, observed in our studies (Fig. 4), does not appear to be achievable with the previously reported delivery strategies for corticosteroids.

A limitation of our study is that only one marker (TNF-α) was used as a measure of pharmacological effects of MP. However, it is likely that MP affects other markers of immune response, in addition to TNF-α, in liver transplant patients. For example, corticosteroids inhibit T-cell-derived and antigen-presenting cell-derived expression of interleukins (ILs) such as IL-1, IL-2, IL-3, and IL-6.[42] However, our LPS model did not result in measurable levels of IL-1β and IL-6 in the perfusate during the relatively short period of the perfusion (120 min).[24] Therefore, we were not able to investigate the effects of MP on these cytokines in this study. Another limitation of our IPRL model is that the isolated organ is perfused with a buffer, instead of blood. Therefore, it lacks blood cells such as leukocytes, which contribute to the inflammatory processes *in vivo*. Nevertheless, previous studies[24,26] from our laboratory have shown that despite these limitations, the model successfully predicts the *in vivo* immunosuppressive effects of corticosteroids after liver transplantation in rats.

Finally, it should be noted that our IPRL model determines the immunosuppressive effects of the systemically-administered drugs only in the liver, without any information about the immunosuppressive activity in other organs. Because we have previously shown that the prodrugs selectively accumulate in the liver, spleen, and kidneys,[28] with no accumulation in most other organs,[28] one may expect an increased local immunosuppression in the spleen and kidneys, in addition to the liver, after systemic administration of the prodrug.

CONCLUSION

In conclusion, the studies presented here show that novel dextran prodrugs of MP with linkers consisting of one (DMP1) or five (DMP5) amino acids exhibit more substantial and sustained immunosuppressive activity in the liver than an equivalent dose of the parent drug. Further, the magnitude and duration of the effect of DMP1 were substantially higher than those of DMP5. A single 5-mg/kg dose of DMP1 persisted in the liver for more than 14 days, gradually releasing the active drug and producing a sustained immunosuppressive activity in the liver. These data suggest that DMP1 may be a suitable candidate for clinical

situations, such as liver transplantation, requiring high concentrations of MP locally in the liver over a sustained period of time. Further pharmacodynamic studies are required to determine the effects of the prodrug in a liver transplantation model.

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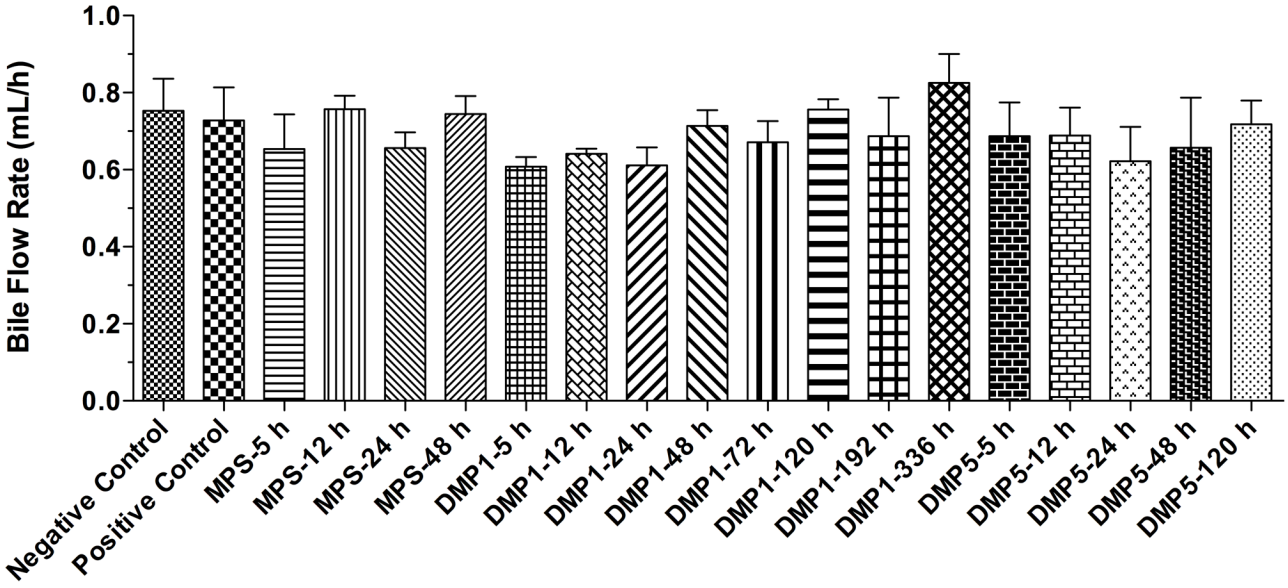

Figure 1.

Average bile flow rates of livers during 2 h of *ex vivo* perfusion. The livers were isolated at different times following pretreatment of animals with a single 5-mg/kg (MP equivalent) dose of MPS, DMP1, or DMP5. The columns and bars represent the mean and SD values, respectively (n = 3–5/group).

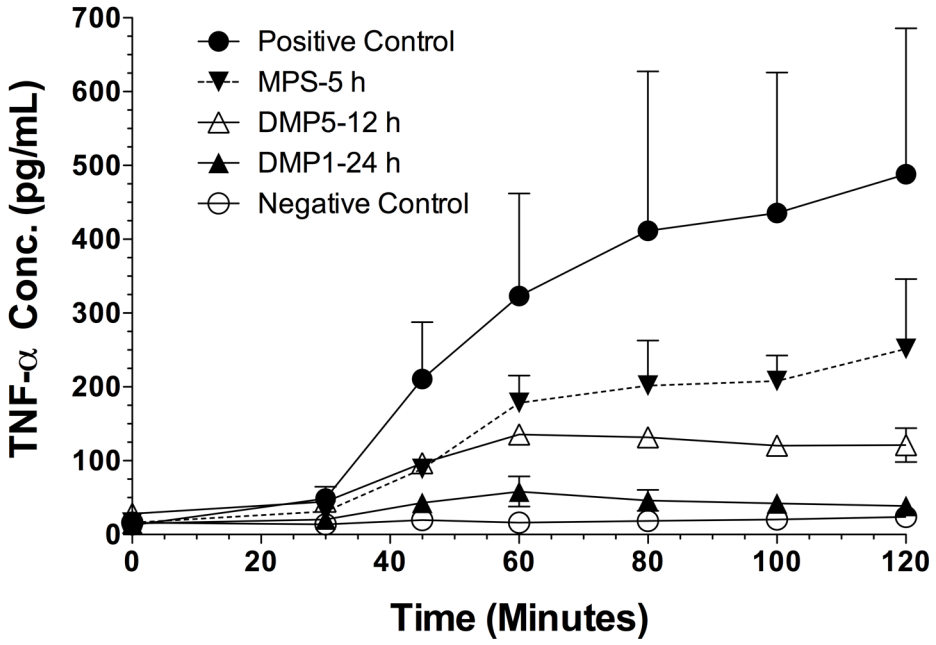

Figure 2.

The concentration-time profiles of TNF-α in the outlet perfusate of livers isolated from untreated animals (negative and positive controls) and the animals pretreated with a single 5-mg/kg (MP equivalent) dose of MPS, DMP5, or DMP1 at 5, 12, or 24 h before the liver isolation, respectively. Except for the negative control group, all the livers were stimulated with 300 μg of LPS during the first 20 min of *ex vivo* perfusion. The symbols and bars represent the mean and SD values, respectively (n = 3–5/group).

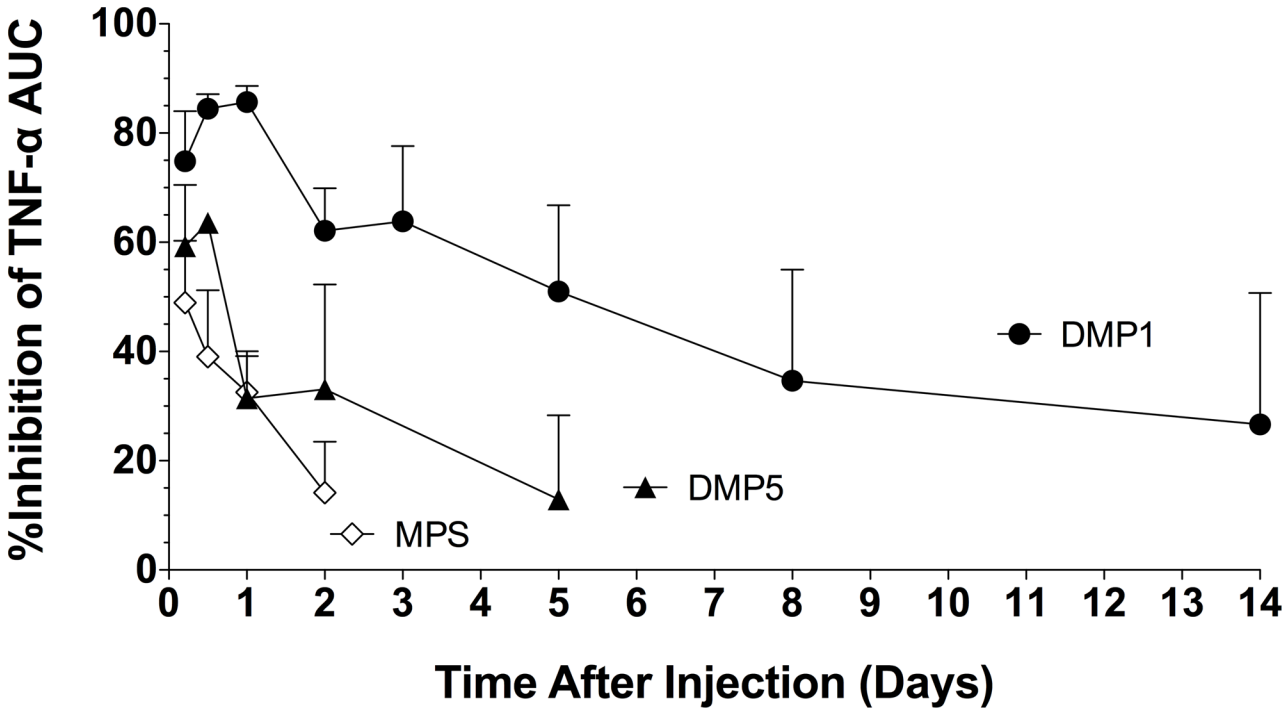

Figure 3.
The time course of the inhibitory effects of a single 5-mg/kg (MP equivalent) dose of MPS, DMP1, or DMP5 on the release of TNF-α into the outlet perfusate of livers stimulated with 300 μg of LPS during the first 20 min of *ex vivo* perfusion. The symbols and bars represent the mean and SD values, respectively (n = 3–5/group).

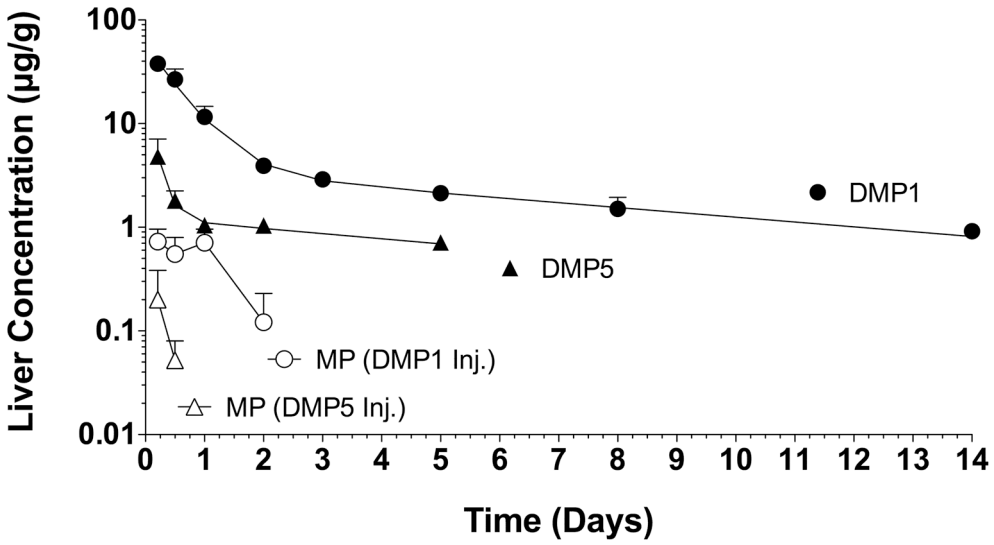

Figure 4.

The hepatic concentration time courses of DMP1, DMP5, and MP regenerated from the prodrugs following a single 5-mg/kg (MP equivalent) dose of the prodrugs. The symbols and bars represent the mean and SD values, respectively (n = 3/group), and the lines for DMP1 and DMP5 are based on the biexponential model with parameters in Table 4.

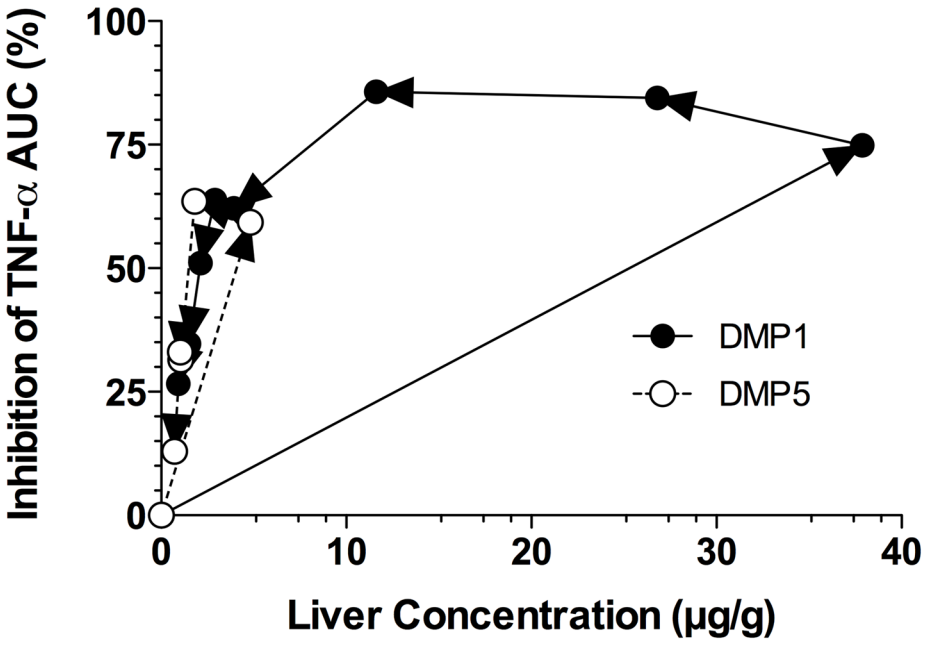

Figure 5.
The effect-hepatic concentration time profiles following a single 5-mg/kg (MP equivalent) dose of DMP1 or DMP5. The symbols and arrows represent the mean and the progression of sampling time, respectively ((*n* = 3–5/group).

Table 1

The AUC values (ng.min/mL) of TNF-α in the Outlet Perfusate of Livers Isolated from Rats Pretreated with a Single 5-mg/kg Dose (MP Equivalent) of MPS, DMP1, or DMP5 Intravenously and Stimulated *Ex Vivo* with LPS (n = 3–5/group)

Treatment	Time after Drug Administration								
	0[a]	5 h	12 h	24 h	2 Days	3 Days	5 Days	8 Days	14 Days
MPS	31.6 ± 12.6	16.1 ± 3.6*	19.2 ± 3.8	21.3 ± 2.4	27.1 ± 2.9	—	—	—	—
DMP1	31.6 ± 12.6	7.94 ± 2.89***	4.90 ± 0.84***	4.51 ± 0.93***	11.9 ± 2.5**	11.4 ± 4.4**	15.5 ± 4.9*	20.6 ± 6.4	23.2 ± 7.6
DMP5	31.6 ± 12.6	12.9 ± 3.6*	11.5 ± 0.6**	21.6 ± 2.4	21.1 ± 6.1	—	27.5 ± 4.9	—	—

[a]Positive control group. The mean ± SD values for the negative control samples (livers without LPS stimulation) were 2.11 ± 0.39 ng.min/mL.

*$p < 0.05$,

**$p < 0.01$,

***$p < 0.001$: Significantly lower than the positive control group based on one-way ANOVA and Dunnett's test.

Table 2

The Pharmacodynamic Parameters of Single 5-mg/kg (MP Equivalent) Doses of MPS, DMP1, or DMP5 Administered Intravenously to Rats

Parameter	MPS	DMP5	DMP1
Peak Time (h)	5	12	24
Maximum Inhibitory Effect (%)	48.9 ± 11.3[a]	63.5 ± 2.0[b]	85.7 ± 2.9[a,b]
Time above 50% Inhibition (h)	< 5	< 24	120
$AUEC_{last}$ (%Inhibition.Day)	59.2 ± 5.3[a,c]	149 ± 26[b,c]	638 ± 72[a,b]

The effect was measured as the %inhibition of TNF-α AUC in the outlet perfusate of the livers stimulated *ex vivo* with LPS.

[a] Significant difference between MPS and DMP1 based on Z test.

[b] Significant difference between DMP1 and DMP5 based on Z test.

[c] Significant difference between MPS and DMP5 based on Z test.

Table 3

The Pharmacokinetic Parameters of Single 5-mg/kg (MP Equivalent) Doses of DMP1 and DMP5 After Intravenous Administration to Rats

Parameter	DMP5 Injection		DMP1 Injection	
	DMP5	Released MP	DMP1	Released MP
Peak Time (h)	5	5	5	5
Maximum Conc. (μg/g)	4.82 ± 2.29[a]	0.200 ± 0.184[b]	37.9 ± 7.2[a]	0.728 ± 0.234[b]
AUC_{last} (μg.h/g)	141± 9[a]	1.38 ± 0.64[b]	1250 ± 64[a]	23.9 ± 3.1[b]
AUC_{∞} (μg.h/g)	306	ND[c]	1490	ND[c]

[a] Significant difference between DMP1 and DMP5 based on Z test.

[b] Significant difference between MP generated from DMP1 and MP generated from DMP5 based on Z test.

[c] ND, Not determined.

Table 4

The Biexponential Parameters of Liver DMP1 or DMP5 Concentration-Time Courses after Single Intravenous Doses of 5-mg/kg (MP Equivalent) of Each Prodrug[a]

Parameter	DMP5	DMP1
C_1 (μg/g)	3.71 ± 2.50[b]	36.4 ± 8.9[b]
C_2 (μg/g)	1.20 ± 0.09[b]	3.59 ± 0.38[b]
k_1 (Day^{-1})	7.13 ± 0.89[b]	1.95 ± 0.00[b]
k_2 (Day^{-1})	0.115 ± 0.010	0.107 ± 0.018

[a] The error values are related to between subject variability.

[b] Significant difference between DMP1 and DMP5 based on Z test.