PEGylated Liposomes Loading Palmitoyl Prednisolone for Prolonged Blood Concentration of Prednisolone

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We investigated the pharmacokinetic behavior of palmitoyl prednisolone (Pal-PLS) and its liposomes with L-α-distearoylphosphatidylcholine (DSPC) and cholesterol (Chol) with or without L-α-distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG 2000) after their intravenous administration in rats. Pal-PLS rapidly disappeared from the systemic circulation and prednisolone (PLS) was regenerated after the administration of DSPC/Chol liposomes. PEGylated liposomes including DSPE-PEG 2000, however, successfully maintained high blood concentrations of Pal-PLS and PLS. The blood profiles of drugs after the administration of liposomal Pal-PLS were analyzed according to a two-compartment model. The larger content of DSPE-PEG 2000 in DSPC/Chol liposomes showed a lower first order elimination rate constant from the central compartment (K_{el}) and clearance (*CL*). The area under the concentration–time curve (*AUC*) of Pal-PLS and PLS in PEGylated liposomes was larger than DSPC/Chol liposomes. The mean resident time (*MRT*) of Pal-PLS and PLS was also prolonged by PEGylated liposomes. Although DSPC/Chol liposomes showed a high distribution of Pal-PLS in the liver and spleen, PEGylated liposomes significantly decreased the liver distribution of Pal-PLS. The biliary and urinary excretions of drugs for 240 min after drug administration were less than 1% of the administrated dose in any formulations. In conclusion, PEGylated liposomes, including Pal-PLS, are useful for maintain the PLS concentration in the blood after intravenous administration.

Key words drug delivery system (DDS); prednisolone; liposome; lipophilic derivative; polyethylene glycol (PEG)

Corticosteroids are frequently used for their immunosuppressive and anti-inflammatory actions. Most glucocorticoids are administrated repeatedly because of their short half-life and are used for not only continuous treatment but also pulse treatment. However, even at moderate doses, the systemic administration of glucocorticoids causes many side-effects, such as diabetes, hypertension, Cushing syndrome, and osteoporosis.[1] Therefore, it is effective for glucocorticoids to be retained in the blood and delivered to target tissues such as inflammatory tissues and the immune system.[2—6]

Liposomes have various advantages as drug carriers such as biodegradability, low *in vivo* toxicity, and the encapsulation of hydrophilic, lipophilic and amphipathic drugs. It was reported that long-circulating liposomes of prednisolone phosphate markedly increased the biological activity and reduced side-effects as compared to its solution after intravenous treatment for arthritis and multiple sclerosis.[2,3] Prednisolone phosphate, however, was difficult to sufficiently incorporate into liposomes because of its high aqueous solubility.

Prednisolone (PLS) is a frequently used glucocorticoid in the clinical field. We previously reported that the low incorporation efficiency of PLS was observed in gel filtration although PLS showed high trapping efficiency by liposomes after ultrafiltration.[7] This indicated that PLS was released easily from liposomes by dilution with elution medium in gel filtration. We therefore newly synthesized palmitoyl prednisolone (Pal-PLS) with high lipophilicity, and successfully prepared liposomes completely incorporating Pal-PLS[7]; however, Pal-PLS was withdrawn out of liposomes in the presence of rat plasma. The effect of rat plasma on withdrawing drug out of liposomes was suppressed by using L-α-distearoylphosphatidylcholine (DSPC) and L-α-distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG 2000).

Therefore, in this study, we investigated the pharmacokinetic behavior of Pal-PLS and its liposomes with DSPC and cholesterol (Chol) with or without DSPE-PEG 2000 after their intravenous administration in rats.

MATERIALS AND METHODS

Materials DSPC (COATSOME MC-8080) and DSPE-PEG 2000 (SUNBRIGHT DSPE-20H) were obtained from Nippon Oil and Fats Co. (Tokyo, Japan). Chol was purchased from Nacalai Tesque Inc. (Kyoto, Japan). PLS was kindly supplied by Shionogi Co. Ltd. (Osaka, Japan). Pal-PLS was synthesized by the method of Teshima *et al*.[7] All other chemicals were of reagent grade and were used as obtained commercially. Phosphate-buffered saline (PBS) was prepared by mixing isotonic phosphate buffer (pH 7.4) with an equal volume of saline.

Preparation of Liposomes The liposomes were prepared by sonication method.[8] DSPC/Chol, DSPC/Chol/1% DSPE-PEG 2000, and DSPC/Chol/10% DSPE-PEG 2000 liposomes were composed of DSPC, Chol, and DSPE-PEG 2000 at a molar ratio of 3 : 2, 3 : 2 : 0.05, and 3 : 2 : 0.5, respectively. After the mixture of lipids and Pal-PLS in chloroform was placed in a round-bottomed glass tube, chloroform was evaporated. The lipid film containing Pal-PLS was further dried *in vacuo* in a desiccator for 4 h. PBS was added to

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the lipid and Pal-PLS film and allowed to hydrate for 24 h at 5 °C. The lipid suspension was vortex-mixed followed by ultrasonic radiation for 3 min at 60 °C. The prepared liposomal formulation was used in this study. We have already reported that the encapsulation efficiency of Pal-PLS into all liposomes was completed without treatment with a separating free drug.[7] The final lipid concentrations of DSPC/Chol, DSPC/Chol/1% DSPE-PEG 2000, and DSPC/Chol/10% DSPE-PEG 2000 liposomes in PBS were calculated as 123.7 mM, 124.9 mM, and 136.1 mM, respectively. The final Pal-PLS concentration was 14 mM. The particle sizes of the liposomes were 42.2±3.5 nm for DSPC/Chol, 72.8±5.1 nm for DSPC/Chol/1% DSPE-PEG 2000, 59.0±4.2 nm DSPC/Chol/10% DSPE-PEG 2000 liposomes in average diameter (standard deviation, determined by a laser electrophoresis analyzer (LEZA-700, Otsuka Electronics Co., Ltd., Osaka, Japan).

Animal Experiments Male Wistar rats (230—290 g) were used throughout the study after anesthetization with an adequate dose of sodium pentobarbital solution. Liposomal Pal-PLS constituted with DSPC/Chol (156.4 mg/kg), DSPC/Chol/1% DSPE-PEG 2000 (163.6 mg/kg), and DSPC/Chol/10% DSPE-PEG 2000 (228.0 mg/kg) was injected into the jugular vain of rats at a dose of 33.6 mg/kg of Pal-PLS (0.5 ml). Blood samples were then collected through femoral vein cannulation at appropriate time intervals (1, 2, 5, 30, 60, 120, 240 min). Bile and urine samples were also collected through biliary and bladder cannulation for 240 min after drug administration. Another group of rats was used for tissue distribution experiments. The rats were sacrificed by an overdose of sodium pentobarbital at 240 min after drug administration and several organs (liver, lung, spleen, kidney, heart, and small intestine) were excised. The organs were weighed, and homogenized in an appropriate volume of 9.8% KCl. The blood, bile, urine, and homogenates were subjected to HPLC assay.

Drug Determination Biological samples (blood, bile, urine, and several tissue homogenates) were separated into two portions for drug determination. The first portions (100 μl) for Pal-PLS were diluted with PBS (2 ml) and extracted with dichloromethane (9 ml) for 15 min. The organic layers (7 ml) were evaporated *in vacuo* and the resultant residues were redissolved in a mixture of ethanol (150 μl) and methanol (150 μl). The mixture was centrifuged at 12000 ***g*** for 10 min and the supernatant was used for HPLC assay. The second portions (100 μl) for PLS were diluted with PBS (2 ml) and extracted with dichloromethane (9 ml) for 15 min. The organic layer was washed with 2 ml of 0.2 M NaOH and 2 ml of water. The organic layer (7 ml) was evaporated *in vacuo*. The residue was redissolved in a mixture of water (150 μl) and methanol (150 μl). The mixture was centrifuged at 12000 ***g*** for 10 min and the supernatant was used for HPLC assay.

HPLC Assay PLS was determined simultaneously with its metabolites by a slightly modified procedure of Cannell *et al*.[9] PLS and Pal-PLS were determined using an HPLC system (LC-10AD, Shimadzu Co. Ltd., Kyoto, Japan) in the reversed-phase mode. The stationary phase was a Cosmosil $5C_{18}$-MS-II packed column (150×4.6 mm for PLS and Pal-PLS, Nacalai Tesque Inc.). A mixture of 2-propanol, acetonitrile, and water (42 : 38 : 20, v/v/v) was used as the mobile phase with a flow rate of 1.0 ml/min for Pal-PLS assay and the retention time of Pal-PLS was 11.9 min. A mixture of methanol and water (57.5 : 42.5, v/v) was used as the mobile phase with a flow rate of 0.55 ml/min for PLS assay and the retention time of PLS was 8.9 min. Drug retention was monitored with a variable wavelength ultraviolet detector (wavelength at 240 nm, SPD-10A, Shimadzu Co. Ltd.).

Pharmacokinetic Analysis The blood profiles of drugs after intravenous administration were analyzed using a two-compartment model. V_c and V_p are the distribution volumes of the central compartment and the peripheral compartment, respectively. K_{el} is the first order elimination rate constant from the central compartment. K_{12} and K_{21} are the first order transfer rate constants between the central compartment and the peripheral compartment, respectively. Pharmacokinetic parameters were calculated by a nonlinear least squares computer program, MULTI.[10] This program was written in BASIC and run on a personal computer (FMV C7/100WLT, FUJITSU, Tokyo, Japan).

Statistical Analysis Statistical comparisons were performed by both analysis of variance and Tukey's multiple comparison test. $p<0.05$ was considered significant.

RESULTS

Blood Concentration of Liposomal Pal-PLS Figure 1 shows the blood concentrations of drugs after the intravenous administration of liposomal Pal-PLS constituted with DSPC/Chol (A), DSPC/Chol/1% DSPE-PEG 2000 (B), and DSPC/Chol/10% DSPE-PEG 2000 (C) in rats. Pal-PLS rap-

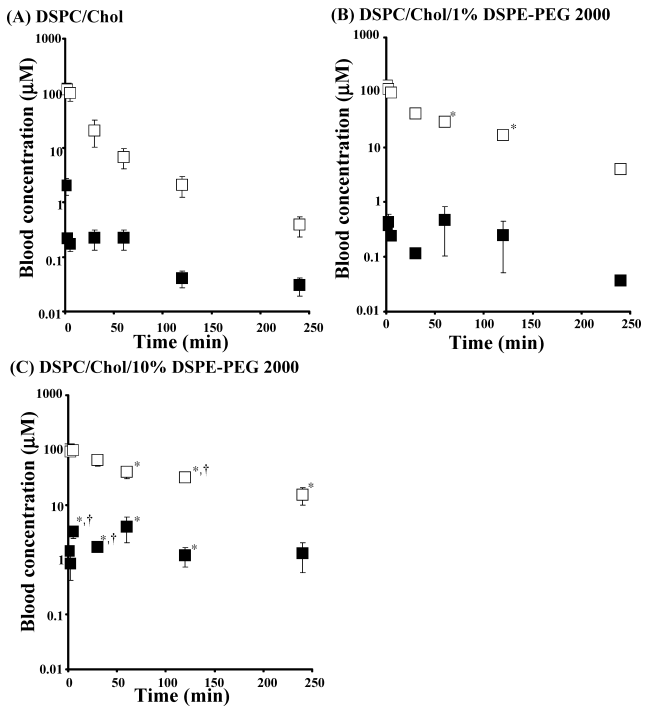
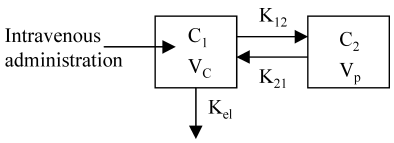


Fig. 1. Blood Concentrations of Pal-PLS (□) and PLS Regenerated from Pal-PLS (■) after the Intravenous Administration of Liposomal Pal-PLS Constituted with DSPC/Chol (A), DSPC/Chol/1% DSPE-PEG 2000 (B), and DSPC/Chol/10% DSPE-PEG 2000 (C) in Rats

Each value represents the average±S.E. of at least three experiments. * Significantly different from liposomal Pal-PLS constituted with DSPC/Chol ($p<0.05$). † Significantly different from liposomal Pal-PLS constituted with DSPC/Chol/1% DSPE-PEG 2000 ($p<0.05$).

Table 1. Pharmacokinetic Parameters of Drugs after the Intravenous Administration of Liposomal Pal-PLS Constituted with DSPC/Chol, DSPC/Chol/1% DSPE-PEG 2000, and DSPC/Chol/10% DSPE-PEG 2000 in Rats



Parameters	Liposomal Pal-PLS constituted with DSPC/Chol	Liposomal Pal-PLS constituted with DSPC/Chol/1% DSPE-PEG 2000	Liposomal Pal-PLS constituted with DSPC/Chol/10% DSPE-PEG 2000
K_{12} (min^{-1})	13.1±6.8	11.6±6.7	6.2±4.3
K_{21} (min^{-1})	36.0±14.7	68.4±19.5	33.4±30.8
K_{el} (min^{-1})	0.25±0.17	0.04±0.01	0.02±0.01
V_c (ml)	39.5±6.4	48.4±8.3	54.2±7.8
V_p (ml)	10.6±5.4	10.9±7.6	18.1±8.0
V_{ss} (ml)	50.1±7.4	59.2±0.6	72.2±4.2
CL ($ml \cdot min^{-1}$)	7.1±3.6	1.6±0.3	1.0±0.3
AUC ($\mu M \cdot min$)	2774±937	5972±550*	9084±815*
	(19±3)	(54±18)	(463±37*,†)
MRT (min)	24.3±6.1	60.3±1.0*	80.5±7.6*
	(72.4±19.0)	(85.4±12.5)	(95.0±21.3)

Each value represents the average±S.E. of at least three experiments. The values in parentheses are pharmacokinetic parameters of PLS regenerated from Pal-PLS. * Significantly different from liposomal Pal-PLS constituted with DSPC/Chol ($p<0.05$). † Significantly different from liposomal Pal-PLS constituted with DSPC/Chol/1% DSPE-PEG 2000 ($p<0.05$).

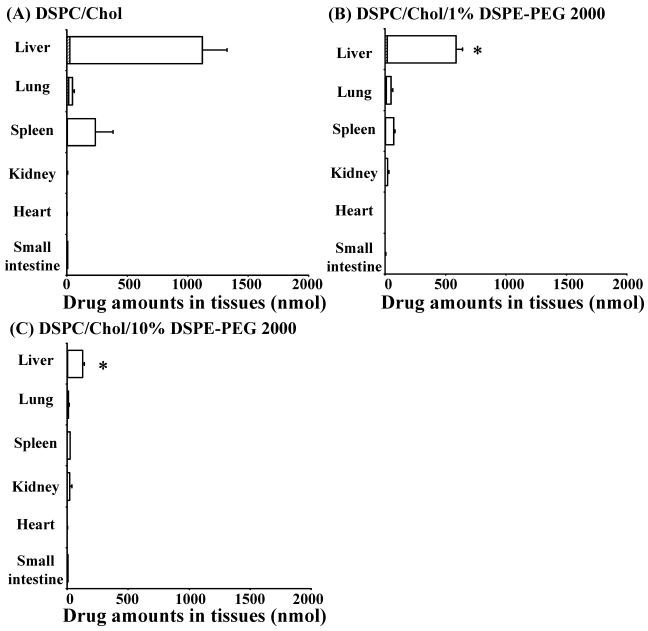


Fig. 2. Tissue Concentrations of Pal-PLS (Open Bars) and PLS Regenerated from Pal-PLS (Hatched Bars) 240 min after the Intravenous Administration of Liposomal Pal-PLS Constituted with DSPC/Chol (A), DSPC/Chol/1% DSPE-PEG 2000 (B), and DSPC/Chol/10% DSPE-PEG 2000 (C) in Rats

Each value represents the average±S.E. of at least three experiments. * Significantly different from liposomal Pal-PLS constituted with DSPC/Chol ($p<0.05$).

idly disappeared from the systemic circulation and PLS was regenerated after the administration of DSPC/Chol liposomes (Fig. 1A). The incorporation of 1 and 10% DSPE-PEG 2000 into DSPC/Chol liposomes significantly increased the blood concentrations of Pal-PLS (Figs. 1B, C). Significantly increased blood concentrations of PLS were observed in DSPC/Chol/10% DSPE-PEG 2000 liposomes compared with DSPC/Chol liposomes and DSPC/Chol/1% DSPE-PEG 2000.

The blood profiles of drugs after the administration of liposomal Pal-PLS were analyzed according to a two-compartment model and the calculated pharmacokinetic parameters are listed in Table 1. DSPC/Chol/DSPE-PEG 2000 (1, 10%) liposomes showed low K_{el} and clearance (*CL*). The area under the concentration–time curve (*AUC*) of Pal-PLS and PLS in the PEGylated liposomes was larger than DSPC/Chol liposomes. PEGylated liposomes also prolonged the mean resident time (*MRT*) of Pal-PLS and PLS compared with DSPC/Chol liposomes.

Tissue Distribution of Liposomal Pal-PLS Figure 2 shows the tissue distribution of drugs (liver, lung, spleen, kidney, heart, and small intestine) at 240 min after the intravenous administration of liposomal Pal-PLS constituted with DSPC/Chol (A), DSPC/Chol/1% DSPE-PEG 2000 (B), and DSPC/Chol/10% DSPE-PEG 2000 (C) in rats. DSPC/Chol liposomes showed a high distribution of Pal-PLS in the liver and spleen (Fig. 2A). The incorporation of DSPE-PEG 2000 into DSPC/Chol liposomes significantly decreased the liver distribution of Pal-PLS (Figs. 2B, C). PLS was hardly observed in the tissues at 240 min after the administration of liposomal Pal-PLS.

Biliary and Urinary Excretion The biliary and urinary accumulation of drugs was examined for 240 min after the intravenous administration of liposomal Pal-PLS constituted with DSPC/Chol, DSPC/Chol/1% DSPE-PEG 2000, and DSPC/Chol/10% DSPE-PEG 2000 in rats (Table 2). The biliary and urinary excretion of drugs for 240 min after drug administration was less than 1% of the administrated dose in any formulations. DSPC/Chol/10% DSPE-PEG 2000 liposomes showed higher biliary and urinary excretion of PLS than DSPC/Chol liposomes.

Table 2. Recovery in Bile and Urine until 240 min after the Intravenous Administration of Liposomal Pal-PLS Constituted with DSPC/Chol, DSPC/Chol/1% DSPE-PEG 2000, and DSPC/Chol/10% DSPE-PEG 2000 in Rats

Parameters	Liposomal Pal-PLS constituted with DSPC/Chol		Liposomal Pal-PLS constituted with DSPC/Chol/1% DSPE-PEG 2000		Liposomal Pal-PLS constituted with DSPC/Chol/10% DSPE-PEG 2000	
	Pal-PLS	PLS regenerated from Pal-PLS	Pal-PLS	PLS regenerated from Pal-PLS	Pal-PLS	PLS regenerated from Pal-PLS
Bile (nmol)	0.5±0.4	1.7±1.0	N.D.	3.5±0.9	0.3±0.1	7.9±3.5
Urine (nmol)	2.6±2.2	1.6±0.4	N.D.	0.2±0.2	0.2±0.1	15.2±11.0

Each value represents the average±S.E. of at least three experiments. N.D.: not detected.

DISCUSSION

In the previous study, we newly synthesized Pal-PLS with high lipophilicity, and successfully prepared liposomes completely incorporating Pal-PLS.[7] Furthermore, the incorporation of DSPE-PEG 2000 into liposomes could inhibit Pal-PLS release from the liposomes in the presence of rat plasma. Liposomal Pal-PLS may be useful for prolonging the blood concentration of PLS and targeting PLS to the reticuloendothelial systems.

We have reported the blood concentrations of drugs after the intravenous administration of PLS and Pal-PLS solutions in a polyethylene glycol 400.[11] PLS and Pal-PLS were disappeared from blood in a two phase mode at half-lives of 8.3 min and 2.2 min, respectively. PLS was rapidly regenerated according to the disappearance of Pal-PLS. Pal-PLS showed a significantly higher accumulation than PLS in the liver and lung.

Silvander *et al.*[12] reported that DSPC/Chol liposomes highly incorporated various fluorescent dyes compared with egg yolk phosphatidylcholine (EggPC)/Chol liposomes. Senior *et al.*[13] also demonstrated that DSPC/Chol liposomes showed longer half-lives than EggPC/Chol liposomes after their intravenous injection into mice. Therefore, we prepared DSPC/Chol liposomes incorporating Pal-PLS and examined the pharmacokinetics of Pal-PLS and PLS after their administration; however, Pal-PLS rapidly disappeared from the systemic circulation and PLS was regenerated at low levels. This rapid elimination of Pal-PLS was explained by the large accumulation of Pal-PLS in reticuloendothelial systems such as the liver and spleen. Drug accumulation in the liver and spleen is generally observed for liposomal formulations irrespective of the loaded contents.

Liposomal Pal-PLS prolonged its half-lives in blood and enhanced its liver accumulation by approximately 4 times compared with Pal-PLS solution. It is worthy to note that the blood half-lives of Pal-PLS was shorter than that of liposomes which was reported by Wang *et al.*[14] Because Pal-PLS was released from liposomes and was converted to PLS in blood, Pal-PLS must disappear from blood faster than liposomes themselves. Another possibility is impairment of liposomal stability in blood by insertion of Pal-PLS into the liposomal membrane.

The surface modification of liposomes with the hydrophilic polymer PEG has been a major advance in drug delivery applications due to the ability of this polymer to reduce protein binding and the plasma elimination of liposomes.[15—17] Pal-PLS incorporated into PEGylated liposomes constituted with DSPC/Chol/1% DSPE-PEG 2000 and DSPC/Chol/10% DSPE-PEG 2000 successfully maintained high blood concentrations and enhanced the AUCs of Pal-PLS and PLS. Increased *AUC* by PEGylated liposomes was caused by the decreased *CL* of Pal-PLS, which suppressed the accumulation of Pal-PLS in the liver. DSPC/Chol/10% DSPE-PEG 2000 liposomes enhanced the AUCs of Pal-PLS and PLS by 3.3 times and 24.4 times, respectively, compared with DSPC/Chol liposomes. In particular, DSPC/Chol/10% DSPE-PEG 2000 liposomes showed prolonged concentrations of PLS in the range of 1—10 μM.

In the previous study, Pal-PLS was easily hydrolyzed to PLS in rat plasma and liver homogenates.[11] Carboxylesterase in the blood and various tissues, especially liver, may play a role in these catalyses. PLS in the blood must be mainly produced from liposomal Pal-PLS in the blood and liver and its rate and amount were influenced by various factors such as distribution and stability of liposomes, and release and conversion of Pal-PLS in the blood and liver. Large central distribution volume and low clearance of Pal-PLS may cause to high concentrations of PLS in the blood after administration of DSPC/Chol/10% DSPE-PEG 2000 liposomes.

Biliary and urinary excretions of Pal-PLS and PLS after administration of liposomes were very small (less than 1% of dose) and varied values as shown in Table 2. PLS in the blood must be excreted in the urine and PLS in the liver and blood must be excreted in the bile. The liposomal Pal-PLS highly accumulated in the liver (DSPC/Chol/1% DSPE-PEG 2000) might show the high bile excretion of PLS although the liposomal Pal-PLS highly maintained in the blood (DSPC/Chol/10% DSPE-PEG 2000) might show the high urine excretion of PLS. Further studies about distribution of liposomes, interaction between drugs and liposomes, and non-linear behavior of PLS at low concentrations are necessary to investigate detail behaviors of liposomal Pal-PLS and PLS.

PLS is extensively used in the clinical treatment of autoimmune diseases, renal diseases, hepatic diseases, and allergic diseases.[18] A dose of 5—60 mg of prednisolone or the equivalent is usually given daily, in divided amounts, although much larger doses may on occasion be required. A hydrophilic corticosteroid, prednisolone succinate, is also administered intravenously at a dose of 5—60 mg to treat patients with severe asthma and shock. After an intravenous injection of 20 mg prednisolone succinate, the maximum blood concentration and half-life of PLS are 481±81 ng/ml (1.3±0.2 μM) and 3.17±0.44 h, respectively. PEGylated lipo-

somal Pal-PLS is useful to maintain PLS concentrations in the blood after intravenous administration. Long-circulating liposomes of prednisolone phosphate have been reported to show increased therapeutic efficiency and decreased side-effects in murine arthritis, multiple sclerosis, and IgA nephropathy.[2—4] Our formulations using chemical and pharmaceutical modification are expected to be used clinically with the right balance of efficacy and side-effects.

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