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Research Paper

Influence of Plasma Cholesterol and Triglyceride Concentrations and Eritoran (E5564) Micelle Size on its Plasma Pharmacokinetics and *Ex Vivo* Activity Following Single Intravenous Bolus Dose Into Healthy Female Rabbits

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Purpose. Eritoran (E5564) is a glycophospholipid that acts as a toll-like receptor 4 (TLR4) antagonist that is being tested as a treatment for severe sepsis and septic shock. In the blood, eritoran binds to plasma lipoproteins altering its pharmacokinetic and pharmacodynamic (PD) effects *in vivo*. The purpose of this study was to determine the influence of changes in plasma cholesterol and triglyceride concentrations on the plasma pharmacokinetics and *ex vivo* activity of eritoran following single intravenous bolus dosing of eritoran to healthy female rabbits fed either a regular chow diet or a cholesterol-enriched diet. This was done with eritoran administered as stable micelle formulations of mean hydrodynamic diameters of 8 or 27 nm).

Methods. Female New Zealand White rabbits were fed a standard diet for 7 days and then randomly assigned either a regular chow diet [regular-diet (n=9)] or a cholesterol-enriched diet [cholesterol-diet (n=12)] for an additional 7 days. Following feeding of these diets a single intravenous bolus dose of eritoran (0.5 mg/kg) formulated into either "small micelles" (8 nm in diameter) or "large micelles" (27 nm in diameter) was administered to regular-fed and cholesterol-fed rabbits. Serial blood samples were obtained prior to eritoran administration and at the following times post injection: 0.083 (5 min), 1, 2, 4, 8, 10, 24, 48 and 72 h. Plasma was analyzed for eritoran concentrations using LC/MS/MS. Total plasma cholesterol (TC) and triglyceride (TG) levels were quantified using enzymatic kits. Plasma eritoran pharmacokinetic (PK) parameters were estimated by non-compartmental analysis using the WinNonlin nonlinear estimation program. To analyze PD activity, whole blood obtained at 0.083 (5 min), 2, 24, 48 and 72 h following eritoran administration was assessed for *ex vivo* activity by measuring the ability of 1 and 10 ng/ml LPS to elicit TNF-α release.

Results. Total plasma cholesterol and triglyceride levels were significantly higher in cholesterol-fed rabbits compared to the rabbits fed a regular chow diet. Diet had no effect on the estimated plasma PK parameters. However, PD activity of both small and large micelle eritoran as measured by an *ex vivo* challenge dose of 1 ng/ml LPS was reduced in blood of cholesterol-fed rabbits compared to normal-fed rabbits. Comparison of PK parameters for small and large micelles indicated that small micelles had increased $AUC_{0-72\ h}$, decreased plasma clearance and increased initial concentration (measured at 5 min post administration) compared to the large micelle formulation. Consistent with this observation, eritoran formulated into small micelles had significantly greater *ex vivo* activity than large micelles and was independent of TC and TG concentrations.

Conclusions. These findings suggest that plasma pharmacokinetics and activity of eritoran maybe influenced by eritoran micelle size and plasma TC and TG concentrations.

KEY WORDS: cholesterol; eritoran; E5564; micelle particle size; pharmacokinetics; rabbits; TNF-α; triglycerides.

INTRODUCTION

Earlier work using [^{14}C]E5564 [eritoran] (3–5,14,16) as well as an analogue ([^{14}C]E5531) (1,9,11,13) suggested that these types of molecules interact with lipoproteins quickly (within 5 min) after addition to human serum at 37°C *in vitro*. Association is mainly with high density lipoproteins (HDL) and with no measurable redistribution between different lipoprotein fractions (21,22). Furthermore increases in plasma cholesterol (TC), triglycerides (TG),

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protein, triglyceride-rich lipoproteins [(TRL; comprised of chylomicrons and very low density lipoproteins [VLDL]) and low-density lipoproteins (LDL)] from different individuals significantly increased partitioning of E5531 into TRL/LDL—while increases in HDL protein significantly increased partitioning of E5531 into HDL (22).

A phase I study (E5564-A001-001) established that after 30-min intravenous infusions of 500–3,500 μg of eritoran, pharmacodynamic (PD) activity was relatively short-lived compared to its pharmacokinetic half-life (23). In another phase I study of a 72-h infusion of eritoran into healthy volunteers, (15) it was determined that eritoran partitioned predominantly (approximately 60%) into HDL in plasma and demonstrated some interaction with other plasma components. This observation may be relevant to the discussion of PD activity, since *in vitro* studies have determined that eritoran partitioned into lipoproteins is deactivated without being degraded. And while the association with LDL and TRL did not readily inactivate eritoran, association with HDL results in a time-dependent inhibition of the activity (12,15). Higher plasma concentrations of eritoran appear to overcome this apparent deactivation of the drug. Following a 72-h intravenous infusion of 500 μg/h and 3500 μg/h eritoran the *ex vivo* drug activity is detectable for up to 72 h post infusion. Under these conditions up to 1,560 and 8,770 ng/ml of eritoran were detected in the non-HDL fractions. The PD activity, as measured with the *ex vivo* response to endotoxin challenge was found to correlate with the plasma concentration. Because partitioning of eritoran into the TRL fraction increases with the drug's concentration in plasma, it is possible that subjects having higher TRL content may have more persistently active eritoran. However, this has yet to be determined, as antagonistic potency of eritoran is likely to be due to a complex interaction between target cells, lipoproteins, proteins and a variety of other serum components, some of which mediate the activity of LPS at the cell surface. But, insofar as up to 40% of infused eritoran can be found in the non-HDL fraction, it is possible that this fraction retains long-term active material.

Little is known about the plasma pharmacokinetics and activity of eritoran following administration to animals with altered lipid profiles. In addition, the effect of formulating eritoran into micelles of different micelle sizes on pharmacodynamic activity has not been described. Thus, the purpose of this study was to determine the influence of lipid profile on plasma pharmacokinetics and *ex vivo* activity following a single intravenous bolus to healthy female rabbits using two different eritoran micelle sizes. Previous studies in rats and dogs (unpublished data) show that larger micelles of eritoran are cleared from blood more rapidly than smaller micelles, which is similar to findings observed by others upon intravenous injection of lipid particles (2). Previous *in vitro* studies and Phase I clinical studies show that deactivation of eritoran will increase if HDL/LDL ratios increase. Thus, changes in cholesterol-fed animals compared with regular chow fed animals administered the drug may effect drug activity over time. The information obtained from these studies could be utilized to correlate plasma lipid levels, plasma cholesterol concentrations (HDL/LDL ratios) and the drug micelle size to pharmacokinetics as well as *ex vivo* activity of eritoran.

MATERIALS AND METHODS

Experimental Design

Rabbits maintained on a chow diet supplemented with cholesterol or regular chow-fed rabbits were administered eritoran formulations as a 5-min infusion into the jugular vein (the total dose was 0.5 mg/kg). Serial blood samples were obtained prior to and 0.083 (5 min), 0.25, 1, 2, 4, 8, 10, 24, 48, and 72 h after infusion; removal of each 3 ml sample (at the 0.083 to 48 h time points) was followed by replacement of an equal volume of normal saline. Twenty milliliters of blood was removed at the 72 h terminal time point by cardiac puncture. Plasma was harvested and stored at 4°C prior to analysis to prevent any redistribution of drug. In addition, whole blood was collected (1.5 ml) from the 0.083 (5 min), 2, 24, 48 and 72 h time points for determination of *ex vivo* eritoran activity. After the 72 h sample each rabbit was humanely sacrificed with sodium pentobarbital (60 mg/kg) administered intravenously over 2 min through the marginal ear vein.

Methods

Eritoran micelle formulations. Eritoran formulated into small micelles (8 nm mean diameter; Lot No. RTP 000135-SN-56-E) and large micelles (27 nm mean diameter; Lot No. RTP 000135-SN-56-C) was provided by Eisai RTP (Research Triangle Park, NC, USA) as a lyophilized powder. Eritoran was reconstituted in water to make 0.5 mg/ml solutions (15).

Rabbit model. New Zealand female rabbits (3–4 kg) were used in these studies. This is an "ideal model" because no kidney or liver function and hematological profile abnormalities were observed in New Zealand white rabbits when 1 ml blood samples are obtained without significant changes in blood flow (6,7). The operative technique for chronic catheter insertion was modified from that of Walsh and coworkers to include a heparin lock device (17).

Determination of eritoran plasma concentrations. Plasma samples were analyzed for eritoran using a validated liquid chromatography/tandem mass spectrometry (LC/MS/MS) method previously developed (3,15,23). This method was also validated for rabbit blood.

Plasma lipid and lipoprotein analysis. Total plasma cholesterol (Chol) and triglyceride (TG) concentrations were determined using established colorimetric and fluorometric techniques as previously described (20–22). HDL-cholesterol was determined using the Mg sulfate precipitation method and LDL-cholesterol levels were calculated using the Friedwald equation and previously described (10,18,19,22).

Pharmacokinetic analysis. Pharmacokinetic parameters were estimated by non-compartmental analysis using the WinNonlin Professional v5.0.1 (Pharsight: Mountain View, CA). Area under the concentration–time curve ($AUC_{0-72\ h}$) was estimated by the trapezoidal rule.

Ex Vivo E5564 Activity Determination

Ex vivo TNF-α induction assay. Blood was collected from each rabbit in a vacutainer tube which contained sodium EDTA and stimulated with purified lipopolysaccharide (LPS)

reconstituted in Hank's Balanced Salt Solution (HBSS); final concentrations were 0, 1 or 10 ng/ml. The mixture incubated for 3 h at 37°C with gentle shaking. After incubation, plasma was obtained and immediately frozen at −80°C until assayed for TNF-α concentration. At each time point, a sample of un-incubated blood was also aseptically reduced to plasma by centrifugation and frozen to −80°C immediately after drawing for baseline TNF-α values. TNF-α concentrations were determined by ELISA developed by ZeptoMetrix Corporation (description of methodology follows below). Prior to doing this assay dose-dependence for LPS-mediated stimulation of TNF-α synthesis was tested in whole blood using LPS at 1 and 10 ng/ml as previously developed (12,15).

TNF-α ELISA measurement assay. ELISA was used to determine the TNF-α concentration in rabbit plasma samples. Microplates were made using an ELISA construction system. The capture antibody, goat anti-rabbit TNF (BD Biosciences) was diluted in a coating buffer at a concentration of 8 μg/ml. Recombinant TNF standard (BD Biosciences) were prepared in two-fold dilutions from 4,000 to 125 and 0 pg/ml (assay blank) in ZeptoBlock (ZeptoMetrix). Samples were diluted eightfold in ZeptoBlock buffer. All standards and samples were loaded on anti-TNF-α plates at 100 μl per well in duplicate and incubated overnight at room temperature. Plates were washed six times with Plate Wash Buffer (ZeptoMetrix). After washing, detection antibody 23H1.1 biotinylated mouse anti-rabbit TNF monoclonal antibody (BD Biosciences) was diluted to 2 μg/ml in ZeptoBlock buffer and incubated on plates at 100 μl per well for 1 h at 37°C. The plates were washed and streptavidin–horseradish peroxidase conjugate (Jackson Laboratory) was diluted to 0.09 μg/ml in ZeptoBlock buffer and incubated on plates at 100 μl per well for 30 min at 37°C. TMB substrate solution was prepared by diluting TMB substrate 100-fold into Substrate Buffer (ZeptoMetrix). After washing, 100 μl per well of TMB Substrate solution was loaded onto plates and incubated at room temperature for 30 min before the reactions were stopped with 100 μl per well of Stop Solution (ZeptoMetrix). Absorbance was measured at 450 nm with a PowerwaveX plate reader using KC-4 data analysis software (Bio-Tek).

Statistical Analysis

The data sets were assessed for normality and equality of variance, if the assumptions of ANOVA were met then two-way analysis of variance with fixed effects was used with Tukey HSD post hoc tests to identify the means that were significantly different (24). If the assumptions of ANOVA were not met, the non-parametric method used was the Kruskal–Wallis analysis of variance and Dunn's post-hoc test to determine significantly different medians. The α value of 0.05 was set prior to all experiments. All statistics were assessed using SigmaStat version 3.5 (Systat).

RESULTS

At time of dosing, the weight of cholesterol-fed rabbits was not significantly different than that of regular-fed rabbits (3.77±0.35 *vs* 3.21±0.24 kg) yet the TC and TG levels were

Table I. Summary of Lipid Profile and Pharmacokinetic Parameters Measured Following a Single Intravenous Bolus of Eritoran (0.5 mg/kg) Formulated as Either Small or Large Micelle Particles of E5564 to Regular-fed (NL) and Cholesterol-fed (HL) NZ White Female Rabbits

Group	Concentration (mg/dl)				$AUC_{0t-last}$ (μg h ml^{-1})	λ (h^{-1})	MRT_{∞} (h)	Vd (ml kg^{-1})	Cl (ml h^{-1} kg^{-1})
	Total Cholesterol	Total Triglycerides	HDL-Cholesterol	LDL-Cholesterol					
NL; small particles (n=3)	43.5±5.9 A,B	38.6±18.2 C	8.9±3.0 E	26.9±6.0 G,H	75.9±14.7 J,K	0.0577±0.014	17.67±2.98	118.6±11.2 L	6.74±1.20 N,O
NL; large particles (n=6)	36.9±9.5 A	50.9±6.3 C	16±7.1 E,F	10.8±8.2 G	12.8±2.3 J	0.0478±0.008	19.69±2.64	855.3±195.0 M	39.99±7.22 N
HL; small particles (n=6)	119.6±51.3 B	67.9±24.4 C,D	24.3±4.1 F	81.8±43.1 H	102.3±28.1 K	0.0415±0.002	23.61±3.61	125.9±35.5 L	5.21±1.43 O
HL; large particles (n=6)	128.6±64.4 B	106.6±29.5 D	25.5±7.2 F	81.7±67.8 G,H	17.5±7.1 J	0.0468±0.008	19.34±3.8	721±319.1 L,M	33.88±15.95 N

Data presented as the mean ± standard deviation. PK parameters were determined using the non-compartmental analysis (WinNonlin).
Values that show statistical significant differences have been identified with a letter. A unique letter designation implies that the means are significantly different (p<0.05).
In addition, three rabbits received vehicle controls, and blood was obtained at 0.083, 2, 24, 48, and 72 h for the TNF-α release induced by LPS (baseline levels).
HDL: high-density lipoprotein, *LDL*: low-density lipoprotein, *AUC*: area under the concentration–time curve, λ: elimination rate constant, *MRT*: mean residence time, *Vd*: volume of distribution, *Cl*: systemic clearance

significantly higher in cholesterol-fed rabbits (Table I). Eritoran formulated into small micelles had similar plasma pharmacokinetic parameters when evaluated in regular-fed and cholesterol-fed rabbits (Fig. 2a). Similarly, eritoran formulated into large micelles had similar plasma pharmacokinetic parameters within regular-fed and cholesterol-fed rabbits (Fig. 2b). However, small micelles had increased $AUC_{0-72\ h}$ decreased clearance and increased $C_{5\ min}$ compared to eritoran formulated into large micelles. This observation was made in both regular-fed and cholesterol-fed rabbits (Fig. 1a and b, Table I). Consequently, eritoran formulated into small micelles had significantly greater *ex vivo* activity than eritoran formulated into large micelles (Fig. 3a). When small micelles of eritoran were administered to normal diet-fed rabbits inhibition of response to 1 ng/ml LPS was nearly quantitative for the duration of the study (>80% at 72 h), but after similar dosing to hyperlipidemic rabbits, antagonistic activity was absent after just 24 h

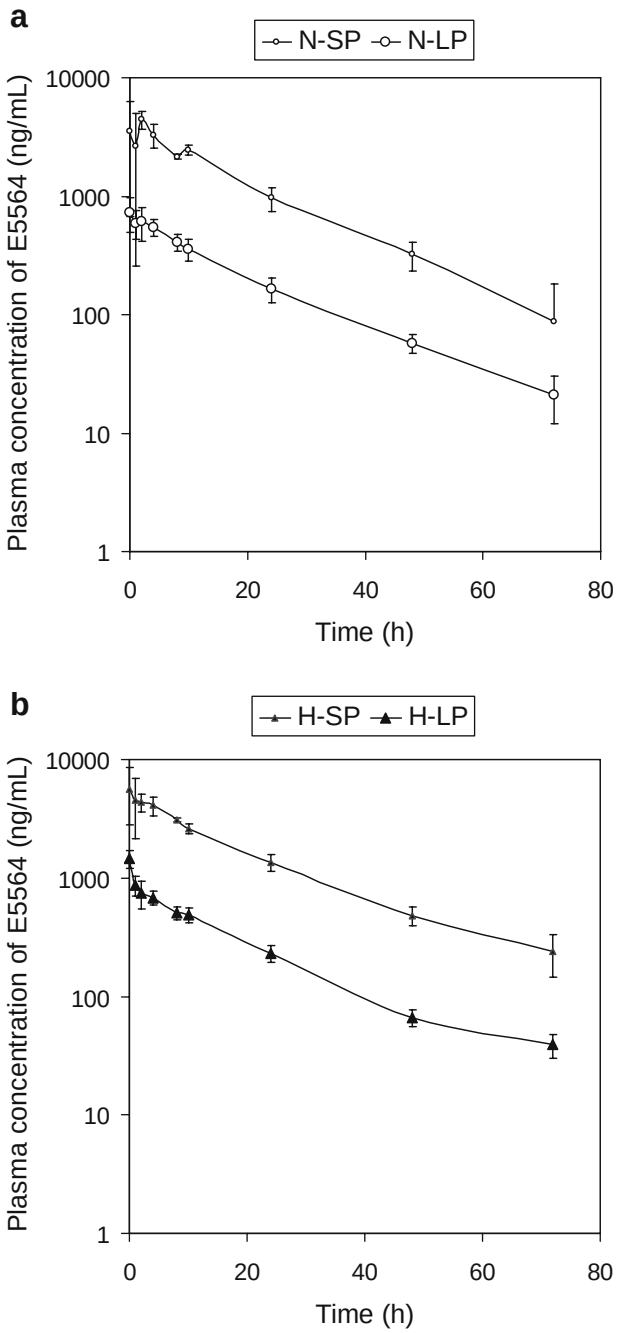

Fig. 1. Log-linear relationship of eritoran plasma concentration *vs* time curve following a single intravenous dose of eritoran formulated as small micelle particles (*SP*) or large micelle particles (*LP*) to **a** regular chow-fed rabbits (*NL*) (*n*=3) and **b** cholesterol-fed rabbits (*HL*) (*n*=6). Mean ± SD.

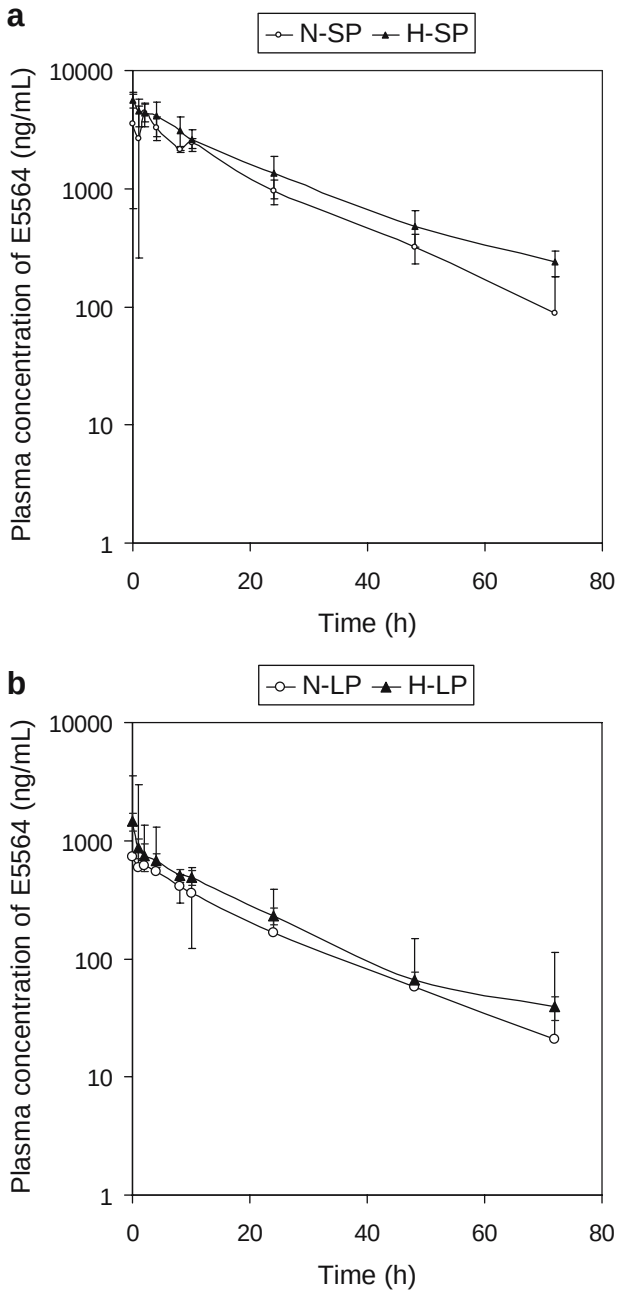

Fig. 2. Log-linear relationship of eritoran plasma concentration *vs* time following a single intravenous dose of **a** eritoran formulated as small micelle particles (*SP*) [regular-fed (*NL*) and cholesterol-fed (*HL*) rabbits] and **b** eritoran formulated as large micelle particles (*LP*) [regular-fed (*NL*) and cholesterol-fed (*HL*) rabbits]. Mean ± SD, *n*=6.

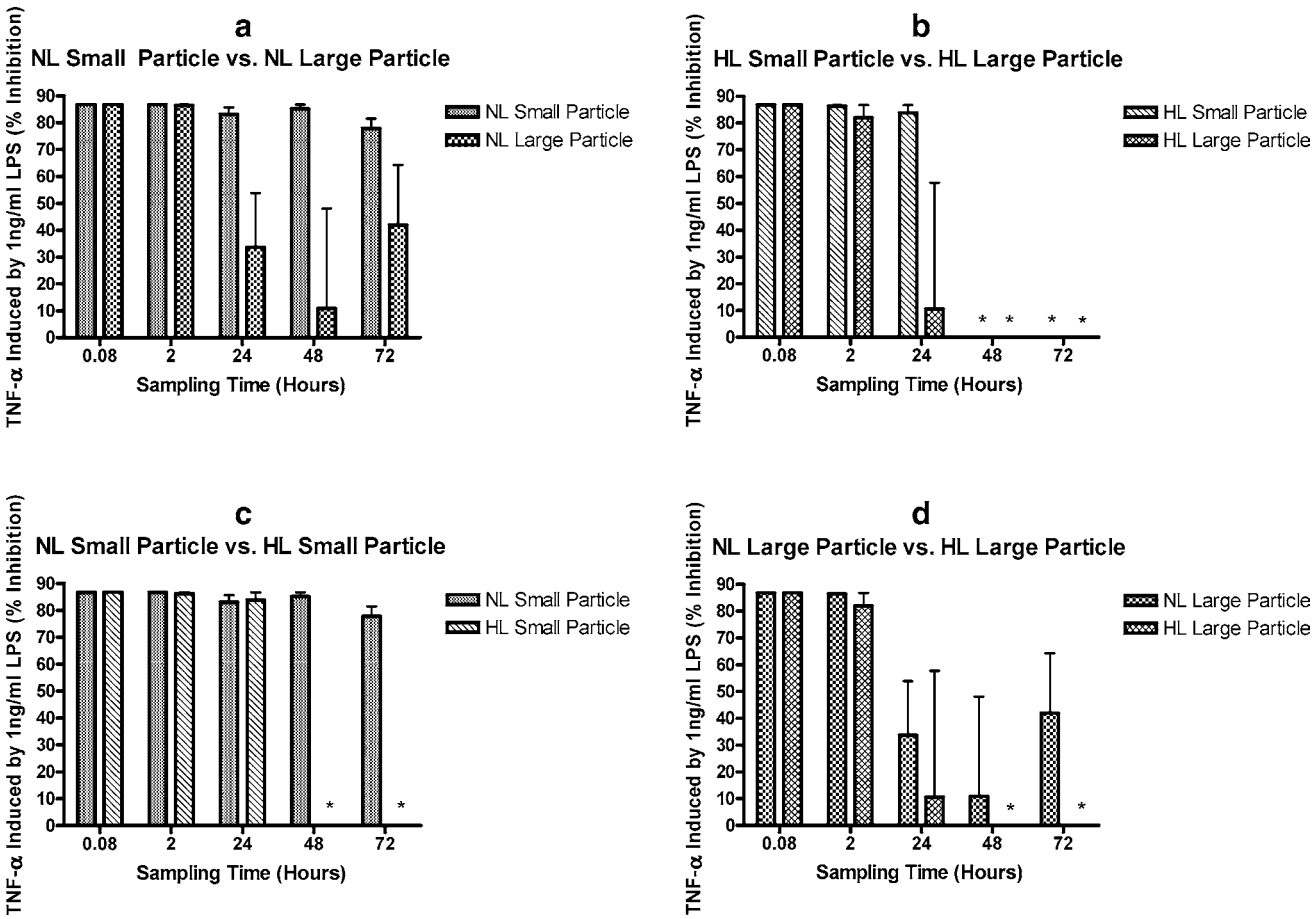

Fig. 3. Percent inhibition of TNF-**α** release by LPS at 1 ng/ml in *ex vivo* blood samples from eritoran (E5564)-treated rabbits. Eritoran was administered as a single intravenous bolus at 0.5 mg/kg as either small micelles (7–9 nm diameter) (*SP*) or large micelles (30 nm diameter) (*LP*) to rabbits with total cholesterol and triglyceride plasma concentrations <100 mg/dl (*NL*) or to rabbits with total cholesterol and triglyceride concentrations >100 mg/dl (*HL*). Blood samples from non-treated and eritoran-treated rabbits were drawn prior to beginning the IV bolus injection or at times after the IV injection as indicated on the *x*-axis and were incubated in triplicate with 1 ng/ml LPS for 3 h at 37°C and the plasma was assayed for release of TNF-α as described in the "Materials and methods" section. Data is presented as the mean ± SEM. Statistically significant differences ($p<0.05$) were observed at the 24 h between small and large particles in **a** and **b**. *Asterisk* indicates that no inhibition was observed.

(Fig. 3c). Similar differences in the PD of large micelles of eritoran in normal-diet and high-lipid diet-fed rabbits were difficult to detect as overall loss of activity was greater (quantitative inhibition was lost between 2 and 24 h post infusion), however, a trend in increased activity in normolipidemic rabbits was observed (Fig. 3d). Determination of any differences in *ex vivo* activity between normal and hyperlipidemic rabbits using a higher LPS challenge dose (10 ng/ml LPS) were not obvious as activity decreased rapidly after 2 h.

Finally, assays using high and low challenge doses of LPS support a dose-dependence for drug activity against the doses of LPS tested. Comparison of Fig. 3c and 4c indicate that at 48 h post administration, small micelles of eritoran quantitatively inhibited the lower challenge dose of endotoxin, but inhibited the higher LPS dose by only 25%. In addition, similar assays carried out with large micelles of eritoran indicated that inhibition was quantitative against the low-challenge dose of LPS at 2 h, but only 75% effective against the higher LPS challenge dose. It is noted that diet alone (regular-fed *vs* cholesterol-fed) did not alter the ability of LPS to elicit TNF-α release (data not shown)

DISCUSSION

The purpose of this study was to determine the influence of changes in plasma TC and TG concentrations and micelle size on the plasma pharmacokinetics and *ex vivo* activity of the new TLR4 antagonist eritoran following a single intravenous bolus dose in healthy female rabbits fed either a regular chow diet or a cholesterol-enriched diet.

We observed that as long as equivalent micelle sizes were administered, no statistically significant differences were observed in eritoran plasma concentration time course plots or calculated pharmacokinetic parameters between regular-chow fed and cholesterol-fed rabbits (Table I and Fig. 2a and b) over the range of lipoprotein concentrations observed. Only minor differences in *ex vivo* activity were observed when eritoran formulated into a larger micelle particle was administered to cholesterol-fed rabbits compared to regular-fed rabbits (Figs. 3 and 4), while more robust differences in *ex vivo* activity were seen when eritoran was formulated into a smaller micelles. At 48 and 72 h time points following eritoran administration there was a signifi-

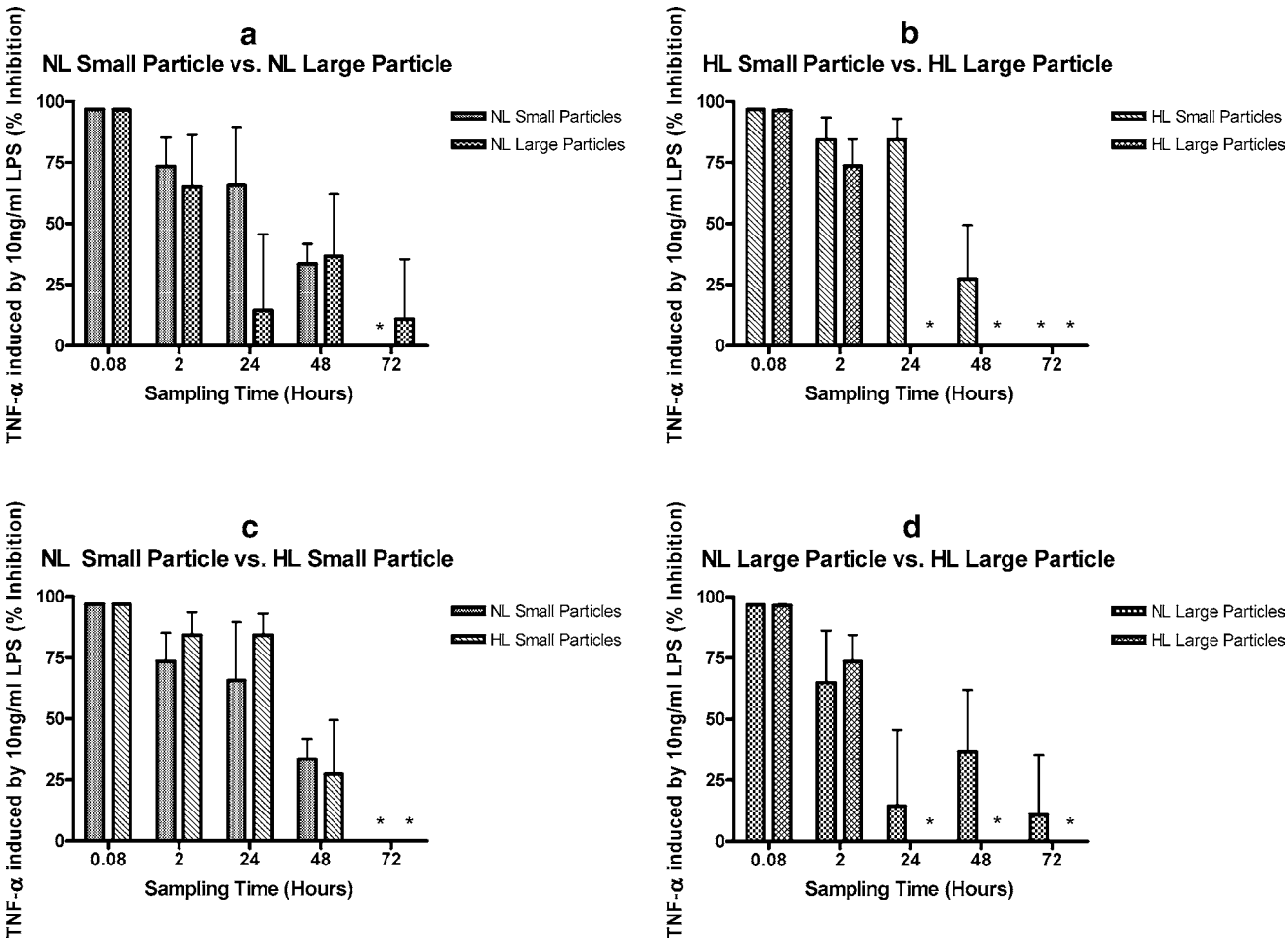

Fig. 4. Percent inhibition of TNF-**α** release by 10 ng/ml of LPS small micelles (7–9 nm diameter) (*SP*) or large micelles (30 nm diameter) (*LP*) of eritoran were administered by intravenous bolus infusion at 0.5 mg/kg to regular-chow fed rabbits (*NL*) or cholesterol-fed rabbits (*HL*) and blood samples were drawn at the times indicated on the *x*-axis, incubated in triplicate with 10 ng/ml LPS for 3 h at 37°C and the plasma was assayed for release of TNF-α as described in the "Materials and methods" section. Data is presented as the mean ± SEM of the triplicate determinations made at each time point in six rabbits. Statistically significant differences ($p<0.05$) were observed at 24 and 48 h between small and large particles in **a** and at 24 h in **b**. Statistically significant differences ($p<0.05$) were observed at 48 and 72 h between regular-fed (*NL*) and cholesterol-fed (*HL*) rabbits administered small particles at 48 and 72 h in **c**. *Asterisk* indicates that no inhibition was observed.

cant loss in antagonistic activity when tested against 1 ng/ml LPS; in samples from cholesterol-fed rabbits compared to regular-fed rabbits (Fig. 3c). This loss of activity at the later time points may be the result of the persistent association of eritoran with HDL. The cholesterol-fed rabbits have approximately a threefold increase in HDL-cholesterol and a threefold to eightfold increase in LDL cholesterol concentration compared to the chow-fed rabbits (Table I) which could result in a greater concentration of drug bound to HDL resulting in its deactivation. This greater concentration may be further explained by the positive correlation between the available HDL surface area and the percentage of drug associated with HDL (data not shown). However, differences in inhibitory activity over time were less clear when blood samples were stimulated with 10 ng/ml of LPS, likely due to the fact that higher concentrations of eritoran are required to block TNF-α release triggered by 10 ng/ml of LPS (Fig. 3). These findings confirm that the sensitivity of the assay at measuring these differences depend on challenge dose used, supporting previous observations that greater concentrations of eritoran are required to inhibit higher concentrations of LPS (23). With respect to a biological relevance of the level of antagonism needed for *in vivo* endotoxin exposure in humans, it is noteworthy that plasma endotoxin concentrations are generally at pg/ml levels, but extreme values can reach 1–10 ng/ml (8).

In a second set of studies, we hypothesized that eritoran formulated into small micelles would have lower plasma clearance resulting in higher eritoran plasma concentrations *vs* eritoran formulated into large micelles, similar to what we have observed in studies in rats and dogs (unpublished data). This increased eritoran plasma concentration would result in greater *ex vivo* activity compared to the larger micelle particle formulation. As reported in Figs. 3 and 4, activity of small micelles of eritoran was significantly better than that of large micelles of eritoran in both regular chow fed and cholesterol fed rabbits. These findings may be directly attributed to the higher eritoran plasma concentrations observed in animals administered small micelles compared to those animals administered large micelles. The higher concentrations achieved abide by the theory that drug delivery vehicles with smaller particles sizes are less recog-

nized by liver Kupffer cells (2) This results in lower eritoran clearance (Table I) and higher eritoran plasma concentrations (Figs. 1 and 2) for the small micelles.

In summary, the findings of the present study show that in the rabbit eritoran plasma pharmacokinetics may be influenced by micelle size and that the resulting differences in plasma concentrations impact the observed *ex vivo* activity of eritoran. In addition, changes in plasma cholesterol and triglyceride concentrations from normal to hyperlipidemic states also impact drug activity, but to a lesser extent. Information obtained from these studies could be utilized to correlate the *in vivo* relationship between plasma lipid levels to pharmacokinetics, *ex vivo* activity and micelle size of eritoran.

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