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Inhibition of Endotoxin Response by E5564, a Novel TLR4- Directed Endotoxin Antagonist

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Abstract

E5564 is a second-generation synthetic lipodisaccharide designed to antagonize the toxic effects of endotoxin, a major immunostimulatory component of the outer cell membrane of Gram-negative bacteria. *In vitro,* E5564 dose-dependently (nM concentrations) inhibited LPS-mediated activation of primary cultures of human myeloid cells and mouse tissue culture macrophage cell lines as well as human or animal whole blood as measured by production of tumor necrosis factor-α (TNF-α) and other cytokines. E5564 also blocked the ability of Gram-negative bacteria to stimulate human cytokine production in whole blood. In vivo, E5564 blocked induction of LPS-induced cytokines and LPS or bacterial-induced lethality in primed mice. E5564 was devoid of agonistic activity when tested both in vitro and in vivo, and has no antagonistic activity against Gram-positive-mediated cellular activation at concentrations up to 1 μM. E5564 blocked LPS-mediated activation of NF-κB in TLR4/MD2 transfected cells. In a mouse macrophage cell line, activity of E5564 was independent of serum, suggesting that E5564 exerts its activity through the cell-surface receptor(s) for LPS, without the need for serum LPS transfer proteins. Similar to E5531, another lipid A-like antagonist, E5564 associates with plasma lipoproteins causing low concentrations of E5564 to be quantitatively inactivated in a dose and time-dependent fashion. However, compared to E5531, E5564 is a more potent inhibitor of cytokine generation, and higher doses retain activity for durations likely sufficient to permit clinical application. These results indicate that E5564 is a potent antagonist of LPS and lacks agonistic activity in human and animal model systems making it a potentially effective therapeutic agent for treatment of disease states caused by endotoxin.

The innate immune response has been described as being composed of inherent abilities to respond to both Gram-positive and Gram-negative bacteria, as well as fungi and other pathogens. Response to Gram-negative bacteria is driven, at least in part, by recognizing and responding to endotoxin (lipopolysaccharide or LPS), a major constituent of the outer membrane of Gram-negative bacteria. LPS is recognized by divergent pathways. One pathway utilizes natural antibodies (Reid et al., 1997) and lipoproteins (Wurfel et al., 1995; Wurfel and Wright, 1995; Hailman et al., 1996; Vreugdenhil et al., 2001) to neutralize and clear LPS. And a second pathway triggers a vigorous and complex inflammatory response involving cellular activation through lipopolysaccharide binding protein (LBP), cell-surface bound CD14, and toll-like receptor-4 (recently reviewed by Diks et al.(Diks et al., 2001)). This latter inflammatory response enables a sensitive and robust reaction to LPS, using it as a "sentinel" molecule, signaling the presence of a potentially infectious agent.

In response to blood-borne infection in mammals, LPS is detected by cells such as monocytes, macrophages, and hepatic Kupffer cells, triggering them to produce a large variety of cytokines, and other cellular mediators (Burrell, 1994)(Fiuza and Suffredini, 2001) that can protect the host. However, during and after bacterial killing or when translocated from the lumen of the intestine, LPS unassociated with worsening infection can induce inappropriate (toxic) levels of cellular mediators that trigger pathophysiological events such as hypotension, fever, shock, and coagulopathies (Bone, 1991b; Bone, 1991a; Ulevitch and Tobias, 1995; Morrison, 1998; Norimatsu and Morrison, 1998; Suffredini and O'Grady, 1999) often leading to multiorgan failure and death (Brandtzaeg et al., 2001). Intestinal tract-derived endotoxin has been implicated as the cause of a variety of clinical manifestations such as post-surgical inflammatory response following abdominal aortic aneurysm (Roumen et al., 1993; Lau et al., 2000) and

coronary artery bypass grafting (Martinez-Pellus et al., 1993), hepatic diseases such as alcoholic cirrhosis (Yin et al., 2001), and aggravation of inflammatory diseases such as graft vs. host disease (Cooke et al., 2001), and inflammatory bowel disease (Gardiner et al., 1995).

In order to prevent LPS toxicity, we have investigated the possibility of developing a receptor antagonist to block its activation of cells. Lipid A is the unique fatty-acylated diphosphorylated diglucosamine portion of LPS that is a common element of LPS from most pathogenic bacteria and is its main toxicophore (Galanos et al., 1985a; Galanos et al., 1985b; Takada and Kotani, 1989) making antagonism of the interaction of Lipid A with target cells an attractive target for the treatment of sepsis, bacteremia, septic shock, and other indications. To this end, we have designed a series of synthetic analogues of lipid A (Rossignol et al., 1999). Previously, we have described the synthesis and activity of E5531, an analogue of the lipid A from *Rhodobacter capsulatus* as an antagonist of LPS (Christ et al., 1995; Kawata et al., 1995) and shown that it's antagonistic action involves the cell surface receptor for LPS-previously described as toll-like receptor-4 or TLR4 (Chow et al., 1999).

While E5531 demonstrated potent inhibition of LPS when added to blood in vitro and in vivo, activity decreased as a function of time. This reaction has been shown to be due to interaction of E5531 with plasma lipoproteins (Wasan et al., 1999; Rose et al., 2000).

This report describes the activity of E5564, a second-generation LPS antagonist derived from the structure of *R. sphaeroides* (Rossignol et al., 1999). Compared to E5531, E5564 is structurally and synthetically less complex, yet appears to possess superior activity and pharmacological characteristics. E5564 is an inhibitor of LPS-mediated stimulation of responsive cells in vitro and in vivo as measured by production of cytokines, as well as morbidity and mortality associated with LPS poisoning in animal models.

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Materials and Methods

Reagents

E5564, {α-D-Glucopyranose, 3-*O*-decyl-2-deoxy-6-*O*-[2-deoxy-3-*O*-[(3*R*)-3-methoxydecyl]-6-*O*-methyl-2-[[(11*Z*)-1-oxo-11-octadecenyl]amino]-4-*O*-phosphono-β-D-glucopyranosyl]-2-[(1,3-dioxotetradecyl)amino]-, 1-(dihydrogen phosphate), tetrasodium salt}, formula weight 1401.60) was synthesized by Eisai Research Institute of Boston, Andover, MA. E5564 was dissolved at 6.7 mg/ml in sterile 0.01N NaOH, sonicated for 3 minutes with an ultrasonicator (VW-380; Heat Systems-Ultrasonics Inc., Farmingdale, NY) then diluted to 100 μM in lactose-phosphate buffer containing (per ml): 0.45 mg $Na_2HPO_4 \cdot 7H_2O$, 0.35 mg $NaH_2PO_4 \cdot H_2O$, and 100 mg Lactose; made up in sterile water. The pH was adjusted to pH 7.8 with 1N HCl and this buffered solution of drug stored as aliquots at -20 °C until use. For use, each aliquot was thawed only once, and serial dilutions were made in Ca^{++}, Mg^{++} free Hanks balanced buffer (CMF-HBSS; Gibco Laboratories, Grand Island, NY). For in vivo use, commercially-formulated E5564 was prepared by treatment of E5564 with NaOH as above, followed by neutralization, addition of phosphate-buffered lactose and lyophilized. Vials of 1 mg E5564 were reconstituted with sterile distilled water and diluted with D5W. The following LPS strains were purchased from List Biologicals (Campbell CA): *E. coli* (Serotype 0111:B4; TCA extracted), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella minnesota* (wild type), *Salmonella typhimurium*, *Serratia marcescens*, and *Salmonella minnesota* R595. *Salmonella entertidis* LPS was purchased from Sigma Chemical Co. of St. Louis, MO. *E. coli* lipid A (Serotype 0111:B4, LA-15-PP(506) was purchased from Daiichi Chemical, Tokyo, Japan. Whole bacteria *Enterobacter aerogenes* Lot# E25081; a clinical isolate, and Lot# ATCC13048 (ATCC) were grown to late exponential phase in Mueller-Hinton broth, and *E. coli*

were grown overnight at 37 °C in heart-brain infusion media (Difco laboratories), harvested, washed by centrifugation in Dulbecco's phosphate buffered saline (calcium-magnesium-free), resuspended in distilled deionized water and lyophilized. Each purified strain of LPS or lyophilized bacteria was solubilized in sterile water for injection and stored as 1 mg/ml aliquots [either as purified LPS or 1 mg/ml (dry weight) whole bacteria], at -80°C. For use, thawed samples were sonicated for 1-2 minutes as above immediately before each experiment. Live *E. coli* (E01292) or *S. aureus* (E31290) were similarly grown and prepared, and used without freezing.

NF-κB reporter activity in TLR4-expressing cells

HEK293 cells stably carrying plasmids for TLR4, MD2, and ELAM-1-luciferase were generated as described (Yang et al., 2000) and shown to be responsive to LPS plus CD14 (Hawkins et al., 2002). Cells were seeded in 96-well plates at a density of 20,000 cells/well and maintained in DMEM plus 10% FBS for 24 h. The next day, cells were incubated with the indicated concentration of E5564 in the presence of LPS (100 ng/ml) and soluble CD14 (10 nM) for 18-20h. Steady-Glo reagent (Promega, Inc., Madison, WI) was added to the wells and the amount of luciferase activity in each sample was quantified in a Wallac 1450 MicroBetaTrilux counter (PerkinElmer, Gaithersburg, MD).

Preparation of Human Whole Blood and Cytokine Assays

Induction of TNF-α in human whole blood has been previously described (Rose et al., 1995; Rose et al., 2000). Briefly, the concentrations of antagonists indicated in the text and figures were added as 10 x stocks in 50 µl of 5% dextrose in water (D5W) followed by 50 µl LPS (10 ng/ml final concentration) to 400 µl of heparinized whole blood obtained from normal

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volunteers (18-51 years old; 50- 105 kg.) for a total of 500 µl/well (final concentration of whole blood was 80%). After 3, 4, 6, 9 or 24 hours (as indicated) incubation with gentle shaking at 37°C in a 5% CO_2 atmosphere, plates were centrifuged at 1000 x g for 10 min. at 4° C then plasma was drawn off and frozen at -80° C. Plasma was appropriately diluted and tested for TNF–α or Interleukins-1β, IL-6, IL-8, and IL-10 and using the appropriate human Predicta™ ELISA kit (Genzyme Diagnostics, Cambridge, MA).

Assays to measure inactivation of antagonists in whole blood were done essentially as described above, but parallel samples containing the appropriate dilutions of test compound were incubated at 37C with shaking for 0, 3, or 6 hours prior to adding LPS, then incubated with LPS for three hours and plasma harvested and assayed for TNF-α as described above.

Animal care and handling

All animals used for harvest of *in vitro* tissue samples were housed and cared for according to the Guidelines For Care and Use of Laboratory Animals (U.S.D.A. NIH Publication 86-23). Sprague Dawley male rats were purchased from (Charles River Lab., Wilmington, MA) and 18-22 g C57BL/6J male mice from Taconic, Germantown, NY. Three to ten week old Hartley white, male guinea pigs were purchased from Elm Hill Breeding Lab., Chelmsford, MA. The animal room was maintained at 65°F ± 3, 45 ± 5% relative humidity with a 12 hour light/dark cycle. Animals were fed solid food and tap water *ad libitum.*

All *in vivo* experiments were approved by Animal Care and Use Committee of Eisai Co. Ltd. Eight to 12-week-old C57BL/6 male mice (Japan SLC Inc., Shizuoka, Japan), 4-6-week-old Hartley guinea pig (Charles River Japan Inc., Kanagawa, Japan) and 5-7 week-old Fischer rats (SLC Inc., Shizuoka, Japan) were housed and cared in our laboratories. The animal room was

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maintained at 23 ± 1° C, 45 ± 5% relative humidity with a 12h light/dark cycle. Animals were fed MF as solid food and tap water was given *ad libitum*. In order to increase sensitivity of LPS, animals were primed 10-12 days before utilization by intravenous injection with 1-2 mg/animal of *Bacillus Calmette-Guerin* (BCG; Japan BCG inc., Tokyo, Japan) suspended in pyrogen-free saline.

Murine whole blood assays

Heparinized whole blood was obtained from eight to ten week old Sprague Dawley male rats or 18-22 g C57BL/6J male mice. Mice were "primed" by injecting, iv., with an attenuated, live preparation of Bacille Calmette Guerin (BCG; 2 mg/0.2 ml/tail vein). Blood was collected 10-12 days after the injection of BCG from CO_2- euthanized animals using cardiac puncture into syringes containing sodium Heparin (LyphoMed Inc., Rosemont, IL) and pooled and stored on ice. One hundred sixty microliters of blood were transferred to wells of a 96-well plate, followed by 20 microliters of E5564 and either 20 microliters of HBSS or LPS. The tissue culture plate was then incubated at 37°C, 5% CO_2 for two hours, on a rotating mixer. Samples were centrifuged (900 x g, 10 min, 4°C) and the supernatants frozen for subsequent assay for TNF-α or IL-6 by ELISA. Mouse TNF-α and IL-6 was assayed using ELISA MiniKits, ENDOGEN, Cambridge, MA, TNF-α was measured in rat plasma samples by ELISA using a rat TNF-α from Biosource and IL-6 was measured in rat plasma samples by a proliferative assay using IL-6 dependent B9 cells (LeMay et al., 1990).

Preparation of Peritoneal Macrophages and Incubations with LPS and E5564

Peritoneal macrophages were isolated from rats, mice, and guinea pigs treated with 2 mg of a cell wall preparation from *Mycobacterium bovis* (BCG; Ribi Immunochem Research Inc.,

Hamilton, MT) as described (Kobayashi et al., 1998). Adherent cells were treated with 10 ng/ml *E. coli* LPS and the indicated amount of E5564 was added to cultures of rat peritoneal macrophages to achieve the concentrations indicated. After a three-hour incubation, plates were centrifuged, and the resulting supernatant samples were stored at -80°C until the cytokine assays were performed. Murine IL-6 and TNF-α was measured as described for plasma (above). TNF-α in guinea pig peritoneal macrophage cultures was quantified by cytotoxicity (LeMay et al., 1990), whereas IL-6 was measured by bioassay as described above.

Statistical Analysis

Unless noted, in vitro experiments were done three times using triplicate determinations in each experiment. Mean and standard error of the mean (SE) were calculated using standard calculations available in the Microsoft Excel spreadsheet. The E5564 concentration that inhibited 50% of the induced production of cytokine (the 50% inhibitory concentration; IC_{50}) was calculated by a log-linear interpolation between the two points that span the 50% value. For in vivo studies, statistical analyses between the control groups and groups treated with E5564 were performed by one-way analysis of variance (ANOVA) or the Fisher exact test followed by Tukey's multiple comparison test or Dunnett's multiple comparison test. A value of 5% (2-sided) was considered statistically significant.

Results

E5564 Inhibition of LPS-induced Cytokines in Human Monocytes and Blood

Assays measuring inhibition of TNF-α induced by 10 ng/mL LPS in adherent human monocytes in the presence of 10% human serum (Christ et al., 1995) indicated that the resultant IC_{50} for E5564 in this system was 0.36 ± 0.2 nM.

Similarly, freshly-drawn heparinized human whole blood dose dependently responds to LPS by producing TNF-α and other cytokines. As in other model systems, 10 ng/ml LPS generated a near-maximal response for TNF-α that peaked at approximately 3 h. As shown in Figure 1, response was inhibited 100% by 10 nM E5564 with an IC_{50} of approximately 1 nM. In three assays the mean IC_{50} for inhibition of this response was 1.6 ± 0.3 nM (Table 1).

Inhibition of induction of other cytokines was also tested in whole blood over incubation periods of 4, 6, 9, or 24 hours. As described in Figure 1, IL-6 levels remained elevated throughout the 24-hour incubation period, and over the same 24 hour incubation period, levels of IL-1ß rose to 1077.9 ± 181.4 pg/ml, IL-8 to over 20,000 pg/ml, and IL-10 to over 450 pg/ml.

E5564 (10 nM) inhibited TNF-α and IL-1β production by 100% at all times tested. Similarly, LPS-induced IL-6 production was inhibited greater than 94% by 100 nM E5564, with a mean IC_{50} value of less than 2 nM (Table 1). Complete inhibition of IL-8 production required slightly higher E5564 concentrations of E5564. LPS-induced IL-8 production was inhibited >70-90% by 100 nM E5564 with an IC_{50} value of 13 nM over the 24 hour incubation time. IL-10 production was inhibited 100% by 10 nM E5564, and the resulting IC_{50} of <1 nM E5564 against this cytokine (Table 1).

Potency of E5564 was dose-dependent for agonist. Other in vitro assays in whole blood, indicated that if LPS concentration was reduced 10-fold, a the IC_{50} of E5564 was reduced approximately two to four-fold (data not shown).

The possibility that E5564 demonstrated agonistic activity in human blood was tested by addition of 10 μM E5564 alone for up to 9 hours. In all experiments, resultant TNF-α and/or IL-6 levels with were at or below basal values indicating that E5564 possesses no LPS-like agonistic activity.

Antagonistic Effects of E5564 on TNF-α Production induced by LPS from different strains of bacteria, whole bacteria, and E. coli lipid A

Lipopolysaccharides derived from various strains of Gram negative bacteria induced TNF-α in human blood. These LPSs demonstrated different dose-dependencies, so for comparison purposes, their concentrations were adjusted to stimulate approximately similar amounts of TNF-α to that induced by the standard *E. coli* LPS (strain 0111:B4) at 10 ng/ml (Table 2).

The IC_{50} values (mean ± SE) for antagonism of LPS response by E5564 are also shown in Table 2. For all strains of LPS the IC_{50} for E5564 was between 1.0 and 12.4 nM, indicating that E5564 is a potent antagonist of these different strains of LPS.

As observed with purified LPS, whole *E. coli* or *E. aerogenes* bacteria induced TNF-α (Table 2). E5564 similarly antagonized this activation with IC_{50}s of 0.65 to 1.5 nM, indicating that E5564 can potently block activation by whole Gram- negative bacteria.

TNF-α induced by 10 ng/mL Lipid A was inhibited 77% by 10 nM E5564 and 100% by 100 nM E5564, with an average IC_{50} of 1.2 ± 0.7 nM (Table 2).

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Antagonism of Cellular Activation by Bacteria in Human blood

In order to determine if cellular activation by live bacteria is inhibited by E5564, antagonism of live Gram-negative bacteria (*E. coli*) and live Gram positive bacteria (*S. aureus*) was tested. As shown in Figure 2, E5564 inhibited induction of TNF-α by high doses of Gram negative bacteria, but was inactive against Gram positive (*S. aureus*) bacteria at concentrations up to 1 μM. These results indicate that E5564 is active against cellular activation by Gram negative bacteria, but not Gram positive bacteria (i.e. activation through TLR2).

Time-dependent inhibition of Antagonistic Activity by Serum

Side by side comparisons of E5564 to E5531 (our "first generation" antagonist) indicated that E5564 is nearly 7-fold more potent an inhibitor of TNF-α production than E5531 (IC_{50} for E5564= 1.5± 0.37 vs. 10.4 ± 3.1 for E5531 (n=7 assays).

The effects of interaction of E5564 and E5531 with blood components was evaluated by preincubating a wide range of concentrations of antagonists with whole blood for 0, 3 or 6 hours before addition of LPS. As shown in Figure 3, the apparent IC_{50} of E5564 increased with time of preincubation in blood (apparent IC_{50}= 1.5 ± 0.4 nM at zero time, 5.9 ± 1.8 nM at 3 hours and 7.7 ± 2.5 nM after 6 hours preincubation in whole blood. Corresponding IC_{50} values for E5531 were 10.4 ± 3.1 nM at zero time, 28.8 ± 8.8 nM at 3 hours and 50.5 ± 16 nM after 6 hours preincubation. These results indicate that low concentrations of E5564 maintain their antagonistic activity longer than similar concentrations of E5531. Higher doses of E5564 (e.g. 1 μm) completely inhibited response throughout the entire 6-hour incubation period. These results indicate that even though E5564 is inactivated by blood, it is likely that dosing can be adjusted to maintain effective levels of antagonistic activity over time.

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Antagonistic activity of E5564 in murine and guinea pig macrophages and blood

Mouse peritoneal macrophages incubated with 10 ng/ml *Escherichia coli* endotoxin for two hours released 3315 ± 318 pg/ml TNF-α and 5.0 ± 0.53 ng/ml IL-6. In this assay, E5564 at a concentration of 100 nM inhibited release of TNF-α by 95% and IL-6 by 89%. The IC_{50} for E5564 was 20.4 ± 12.5 nM against TNF-α and 16.6 ± 6.7 nM against IL-6 (Table 3).

In mouse whole blood, survey studies have previously indicated that *ex vivo* induction of TNF-α was not robust or reproducible. However, IL-6 was found to be dose-dependently stimulated by LPS with maximal response at 10 μg/ml LPS, and greatest dose-dependency at 1 to 100 ng/ml LPS. This response was dependent on time of incubation with LPS for only up to two hours with little or no further increase seen thereafter (data not shown). After two-hour incubation with 10 ng/ml LPS, IL-6 concentrations rose to 13 ± 0.18 ng/ml (n=3) compared to 0.158 ± 0.013 ng/ml in samples that were not treated with LPS. E5564 at a concentration of 100 nM inhibited the IL-6 increases by 85%, and the IC_{50} for E5564 was 20.2 ± 7.0 nM in these combined experiments (Table 3).

To test for inhibition of NO production, cultured mouse macrophages (RAW 264.7) were incubated overnight with 10 ng/mL LPS which induced accumulation of more than 20 μM nitrite in the culture medium. E5564 dose-dependently inhibited this induction [IC_{50} = 91 ± 36 nM (mean ± SE; n=5)] with >95% inhibition observed at 1 μM E5564 (data not shown)

Rat peritoneal macrophages stimulated with 10 ng/ml LPS induced 2867 ± 326 pg/ml TNF-α, whereas induction of IL-6 was robust but variable with release of between 23 and 163 ng IL-6 per mL of culture medium. E5564 at 100 nM inhibited TNF-α and IL-6 production by 89%. The IC_{50} for E5564 was 7 ± 5.6 nM for TNF-α and 16.2 ± 17.5 nM (28.6 and 3.9 nM) for IL-6 (Table 3).

E5564 was significantly less active in rat blood than in rat peritoneal macrophages. At a concentration of 1000 nM, E5564 inhibited LPS-induced TNF-α increases by 86%, and the IC_{50} for E5564 was 136 ± 61 nM in these combined experiments. In the same incubations where TNF-α was measured, 10 μM E5564 inhibited the LPS-induced IL-6 production by 70% with an average IC50 of ~2400 nM.

Guinea pig macrophages incubated with 10 ng/ml *Escherichia coli* LPS for three hours, released 1,897 ± 348 pg/ml TNF-α (n=3) and 3.0 ± 0.43 ng/ml IL-6 (n=2). E5564 (10 nM) inhibited LPS-induced TNF-α and IL-6 by >98%, with a resultant IC_{50} for E5564 of 0.30 ± 0.15 nM for TNF-α and 0.5 ± 0.3 nM for IL-6 (Table 3).

Inhibition of LPS induced TNF-α release in vivo

E5564 or vehicle was injected intravenously into BCG-primed mice along with 100 μg/kg of LPS, a lethal dose. One hour after administration, blood was collected and TNF-α levels measured (Figure 4). E5564 administered at 30, 100, 300, or 1000 μg/kg suppressed plasma TNF-α concentrations by 24%, 38%, 81% and 93%, respectively.

BCG-primed guinea pigs were intravenously injected with a lethal dose of LPS (1000 μg/kg) and E5564 or vehicle and blood was collected one hour later for measurement of TNF-α levels (Figure 4). Administration of 10, 30, 100 and 300 μg/kg of E5564 suppressed LPS-induced plasma TNF-α concentrations by 29%, 57%, and 94%, respectively. The ED_{50} for this model system was estimated to be 37 μg/kg of E5564.

Similarly, BCG-primed rats were intravenously administered E5564 or vehicle along with 3 μg/kg of LPS (a non-lethal dose) and blood was collected one hour later for assay of TNF

concentrations (Figure 4). Administration of E5564 inhibited induction of TNF-α by 84%, 97%, and 100%, by 10, 100, and 1000 μg E5564/kg respectively.

Effect of E5564 on LPS-induced lethality in mice

To evaluate the ability of E5564 to prevent LPS-induced mortality, BCG-primed mice were injected intravenously with 100 μg/kg of LPS along with the indicated doses of E5564 or vehicle, and incidence of mortality was monitored for 72h (Figure 5). Whereas the administration of 100 μg/kg of LPS alone resulted in 90% mortality by 72h, co-injection of E5564 significantly and dose-dependently reduced the incidence of mortality.

Effects of E5564 on septic shock caused by bacterial infection in mice

The objective of the study was to examine the use of E5564 in conjunction with the β-lactam antibiotic latamoxef to prevent mortality in mice injected with *E. coli*. A suspension of *E. coli* (3.23×10^7 cfu/animal) was injected intraperitoneally into BCG primed mice. One hour later the mice were injected intravenously with vehicle, E5564 alone (5 mg/kg), latamoxef alone (30 mg/kg), or E5564 and latamoxef together. The incidence of mortality was recorded for 72 hours after infection.

As shown in Figure 6, mortality of the *E. coli*-infected control group (no treatment) reached 90%. Administration of either latamoxef or E5564 alone resulted in a modest reduction in the incidence of mortality. However, simultaneous administration of E5564 and latamoxef suppressed mortality to 20%. These results demonstrate that combined treatment with E5564 and latamoxef is significantly more effective than treatment with antibiotic alone or E5564 alone in reducing the incidence of mortality caused by *E. coli* infection in BCG-primed mice.

Lack of Agonistic activity of E5564 in vivo

E5564 was intravenously injected into BCG-primed animals (3000 μg/kg into mice, 300 μg/kg into guinea pigs, and 100 and 1000 μg/kg into rats) and blood was collected for analysis of TNF-α one hour later. Neither E5564 nor vehicle alone caused any marked induction of TNF, indicating that E5564 is devoid of endotoxin-like activity in this assay.

How Does E5564 work?

Interaction of E5564 at the TLR4 receptor for LPS has been investigated by measuring response to LPS (expression of NF-kB-driven luciferase) by HEK 293 cells made responsive to LPS by transfection with TLR4 and MD2. As shown in Figure 7, luciferase expression in this system is dependent on LPS plus soluble CD14 and inhibited by E5564 (IC_{50}=32 nM). By contrast, E5564 did not inhibit heat-killed *S. aureus*-activation of TLR2- transfected Hek293 cells at concentrations up to 1 uM (J. Chow, unpublished data). These results suggest that E5564 interferes with LPS signaling at the TLR4 receptor and/or soluble CD14.

In addition, because LPS associates with soluble serum receptors such as LBP and sCD14 before interacting with TLR4, these proteins or other soluble serum factors could also be critical "receptor(s)" for E5564 or be necessary for antagonistic activity. To determine if E5564 needs to be present in the culture media and/or serum or if pretreatment of the cell surface can block LPS-mediated activation, RAW 264.7 cells were tested for inhibition of TNF-α release after various drug pretreatment regimens. Pilot experiments indicated that treatment of cells with E5564 followed by rapid, extensive cell washing and addition of LPS along with serum or LBP for three hours resulted in clear dose-dependent inhibition of LPS activity. As shown in Table 4, addition of E5564 in the absence of serum followed by washing (3 times) and addition of serum plus LPS, resulted in IC_{50}s of 0.8±0.1 nM (n=3). If serum is included in this E5564

pretreatment the resultant IC_{50} was unchanged at 1.1 nM (n=2), indicating that serum did not increase or dramatically decrease the potency of E5564. Similarly, purified LBP used in the place of serum had no effect on E5564 activity (data not shown).

To determine if E5564 retained its activity after unbound material was removed from the adherent cells or if it is readily released from the cell surface, cells were treated with different concentrations of E5564 in the absence or presence of serum, followed by washing and incubating for up to 2 hours with and without serum to allow antagonist dissociation. LPS plus serum was then added and cellular stimulation measured by TNF-α release. The resulting IC_{50} values for E5564 were unchanged after 30 minute incubation in serum free medium (0.7±0.1 nM) and increased slightly (2.5 nM) when serum was included in the incubation medium. Extending the "washout" period to two hours in the presence of serum further increased the IC_{50} to 24-25 nM, indicating a slow loss of "surface-associated" antagonistic activity.

Discussion

A variety of pathologies have been attributed to responses to endotoxin even in cases where identification of its source is unclear. In the clinic, antibiotic treatment to control bacterial infection has been reported to result in the release of endotoxin and consequent aggravation of septic shock. While the source of endotoxin during infection by Gram-negative bacteria may appear obvious, endotoxemia has also been reported to occur during Gram positive and fungal infection (Opal et al., 1999). In addition, it has been reported that endotoxin is translocated from the intestinal tract to the splanchnic circulation under a variety of conditions. Perhaps more definitive proof that pathological outcomes are truly due to endotoxin awaits the therapeutic application of an effective endotoxin antagonist.

Mechanism of action of E5564.

It is not clear exactly how E5564 works to block LPS signaling. Since E5564 is a structural analog of the lipid A portion of LPS, it is logical to hypothesize that the antagonist interacts with the same signaling components that interact with LPS such as the soluble serum proteins LBP and sCD14, as well as membrane-associated CD14 and perhaps the TLR4/MD2 receptor complex. In the present study, E5564 blocked LPS/sCD14-induced reporter activity in TLR4/MD-2- expressing HEK293 (Figure 7), but not TLR2-mediated signaling by heat-killed *S. aureus*. These findings indicate that E5564 selectively inhibits LPS signaling via TLR4/MD2. However, a limitation to this model system is that LPS requires the presence of sCD14 for cellular activation, making it difficult to determine whether E5564 blocks LPS binding to sCD14 or TLR4/MD2. Results from experiments described in Table 4 indicated that serum components neither increased nor dramatically decreased the potency of E5564 indicating that they are not critical to E5564 antagonistic activity.

Further support of the hypothesis that interaction of E5564 at CD14 does not play a key role in its activity comes from a previous study by Lien et al. (Lien et al., 2000) describing the activity of novel synthetic acyclic lipid A-like agonists that activate TLR4/MD2 in the absence of CD14. E5564 inhibited the actions of these agonists under serum-free conditions. Taken together, these lines of evidence make it tempting to speculate that E5564 binds to TLR4/MD2 complex thereby blocking LPS binding or transmembrane signaling.

The downstream effect of inhibiting the initial signaling by LPS appears to be an inhibition of all LPS-induced cytokines measured, including TNF-α, IL-1β, IL-6, Il-8, IL-10 and nitric oxide which was measured in cultured cells, whole blood and in vivo.

The likelihood that E5564 does not inhibit the interaction of LPS with soluble receptors may increase its value as a therapeutic agent. Because endotoxemia and sepsis induce acute phase in the liver, plasma levels of proteins such as LBP change dramatically (Opal et al., 1999). Therapy targeted towards these serum proteins would require dosing adjustments to accommodate increased synthesis or turnover of these proteins.

Comparisons of antagonistic potency in cells cultured in 10% serum vs. whole blood allow us to determine if the high concentration of proteins/lipoproteins present in serum inhibit E5564 activity. In all systems but the rat, antagonistic activity of E5564 in cultured cells was within 4-fold that measured in high serum (blood) compared to assays done in low serum conditions (cultured cells or monocytes). This indicates that serum has little or no inhibitory effect on antagonistic activity under these in vitro conditions. However, extended incubations in whole blood demonstrated that activity of E5564 was measurably reduced. Other studies (manuscript in preparation) indicate that like E5531, E5564 is not rapidly metabolized, but binds to lipoproteins, and time-dependently loses antagonistic activity. The observation that lipoproteins

reduce drug activity may explain the poor activity of E5564 in rat blood that has a relatively high lipoprotein content (Segrest and Albers, 1986).

Is Endotoxin the Major Component from Gram Negative Bacteria that Activates Cytokine response in Blood?

It has been postulated that bacterial components other than endotoxin may activate cells in whole blood, and recently, different subtypes of toll-like receptors have been implicated in responses to these different components. However, this differentiation of response to different receptors does not indicate the relative importance of these components in cellular stimulation. E5564 appears to be a specific antagonist for the TLR4 receptor and is inactive against TLR2-directed agonists (Heine et al., 2000). Based on the ability of E5564 to inhibit cellular activation by LPS from a variety of Gram-negative bacteria, whole killed or live bacteria, but not Gram-positive bacteria, it is likely that E5564 is an effective antagonist for activation of blood cells specifically by Gram-negative bacteria. Furthermore, the observation that E5564 is a potent and complete (or nearly complete) inhibitor of Gram-negative bacteria in the bacterial sepsis model helps clarify the role of endotoxin in activation of cells in blood. Effective reduction of inflammatory response and death due to bacterial infection by E5564 indicates that endotoxin or other agents that bind to TLR4 drive response to Gram negative bacteria.

Therapeutic Potential of E5564 as an Endotoxin Antagonist

E5564 is being developed as an endotoxin antagonist for human therapeutic use.

Safe treatment with E5564 requires that it generate no LPS-like response on its own (Rossignol et al., 1999). When added alone to whole blood or injected into BCG-primed animals, E5564 was devoid of agonistic activity (cytokine generation or overt physiological effects) at

concentrations as high as 100 μM in vitro or up to 1000 μg/animal (the highest concentrations and doses tested).

During extended incubation in whole blood, E5564 retained activity better than similar concentrations of the "first-generation" antagonist, E5531. Based on the proposed mechanism of action as a cell-surface antagonist, it is likely that E5564 can completely block cellular activation by LPS. This block is achieved by concentrations of E5564 as low as 10 nM (14 ng/mL) in vitro, and at doses of 1 mg/kg or less in animal models challenged with lethal doses of endotoxin. In animals lethally-infected with *E. coli*, treatment of the infection with antibiotic alone did not prevent death in a majority of the animals, however, combining antibiotic with 5 mg/kg E5564 reduced mortality to one third that of antibiotic alone. It is important to note that in this model, infection and endotoxin exposure began one hour prior to administration of anti-endotoxin therapy differing from our prophylactic treatment models in that endotoxin is present prior to administration of E5564 and representing a situation more like that of human infection. We have previously shown that this model involves profound endotoxemia- especially after antibiotic treatment (Christ et al., 1995; Kobayashi et al., 1998) however, we have not measured plasma endotoxin levels in treated animals in this study because E5564 is a potent activator of the limulus assay, likely due to its structural similarities to Lipid A (manuscript in preparation).

Both our LPS-challenge model and infection model utilize animals that have been sensitized or "primed' to LPS by previous infection with BCG, increasing cytokine response and lowering the threshold lethal dose of endotoxin. All animal models of sepsis and infection have been criticized for their inability to closely mimic human sepsis. However, we believe that the primed model is the most relevant to the study of endotoxin antagonists such as E5564. It is well-known that compared to humans, unprimed rodents such as rats and mice and primates demonstrate a

profound insensitivity to endotoxin, requiring endotoxin doses as high as mg/kg, while humans demonstrate reproducible response to endotoxin at doses as low as 2 ng/kg. This argues that either LPS contributes only weakly to the inflammatory process in animal models, or that response to infection occurs only after the level of infection is very high, representing a process different than that in more LPS-sensitive species such as humans.

Even in "primed" animal models, lethal doses of LPS in are high; approximately 100 μg/kg generating estimated plasma concentrations of ~1 μg/ml. These plasma levels are still >100-fold that found in even the most extreme cases of human sepsis (Opal et al., 1999). Because the dose of E5564 required to protect against LPS is proportional to the LPS challenge dose, studying E5564 in these animal models indicates that E5564 can be a safe and effective antagonist even under these extraordinary conditions. E5564 is approximately 10-fold better in human blood than mouse blood (IC_{50}=1.6 nM in human whole blood; Table 2 vs. ~ 20 nM in mouse whole blood; Table 3). Taken together, comparison of in vitro and in vivo results leads us to believe that in clinical use, we may be able to readily establish plasma concentrations of E5564 that will provide us with a wide margin of efficacyagainst even very high plasma concentrations of LPS.

Complete block of cytokine response by 10 nM E5564 in blood extrapolates to a human dose of ~100 μg in a 70 kg individual. Recent studies have supported this extrapolation by finding that a dose of 100 μg E5564 given to normal volunteers over 30 minutes completely blocks response (signs, symptoms and cytokines) to a dose if 4 ng/kg endotoxin administered at the midpoint of the E5564 infusion (in press).

In vitro and ex vivo assays have found that low concentrations of E5564 time-dependently lose ability to inhibit LPS response. In light of these observations, it is perhaps not surprising that low doses of E5564 demonstrate a time-dependent loss of activity following administration into

normal volunteers. This loss in activity is overcome when E5564 doses are increased (in preparation). Phase I clinical safety and tolerability assays indicate that E5564 is safe and except for the occurrence of phlebitis, well tolerated at doses up to 252 mg administered over 72 hours. At this dose, *in vivo* antagonistic activity is retained for at least an additional 72 hours after discontinuing infusion. This leads us to believe that sufficient therapeutic activity can readily be administered to patients.

In conclusion, we have found that E5564 is a highly-active antagonist of LPS in vitro in human and animal systems. This activity translates into effective in vivo antagonism of endotoxin in animal models resulting in enhancement of survival after challenge with endotoxin or bacterial infection. This consistent, potent, activity combined with a clear lack of "LPS-like" agonistic activity leads us to believe that E5564 can be a safe clinical therapeutic for the treatment of endotoxemia, including sepsis and septic shock.

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Footnotes

Biology Section, (MM, JRR, JB, SK, MP, JC, FG), and Chemistry Section (WJC)

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Legends to Figures

Figure 1. Inhibition of LPS-induced cytokines in human blood.

Upper panel: 10 ng/mL LPS plus the indicated concentration of E5564 were added to human whole blood, incubated for three hours at 37C, and TNF-α assayed as described in the Materials and Methods section. Each point and value in the Figure represents the mean and standard error of triplicate determinations obtained in a single experiment. No measurable TNF-α was observed in samples incubated without LPS. Statistical significance of inhibition (compared to the LPS-only response) was: *$p<0.05$ or **$p<0.001$ by Student's T test.

Lower panel: 10 ng/mL LPS alone (closed symbols) or plus 10 nM E5564 (open symbols) was added to human whole blood and incubated for four, six, nine, or twenty-four hours at 37° C. After the incubation, the blood was centrifuged, and the resulting plasma supernatant samples were assayed for TNF-α (●,○), IL-1ß (◆,◇), IL-6 (▼,▽), IL-8 (▲,△) or IL-10 (■,□). Complete inhibition of release of all cytokines was observed in the presence of 100 nM E5564 (not shown). Incubation of unstimulated blood samples for up to 24 hours induced less than 1% of peak levels for TNF-α, IL-1ß, IL-6 and IL-10 and less than 4% of peak levels of IL-8 from samples containing LPS. Each point and value in the Figure represents the mean and standard error of triplicate determinations. This experiment was performed three times with similar results. Statistical significance of inhibition (compared to the LPS only samples) for the three assays was: $p<0.05$ for all values of IL-8 and IL-6 measured in the presence of 10 nM E5564 and $p<0.001$ for all values of IL-10, IL-1β and TNF-α (except for the 24-hr TNF-α time point) measured in the presence of 10 nM E5564 (Student's T test).

Figure 2. Effects of E5564 on induction of TNF-α by Gram negative and Gram positive bacteria.

The indicated concentration of E5564 was added to heparinized human blood immediately before innoculation with either 1 x 10^5 cfu/ml *E. coli* (E01292;●) or 1 x 10^6 cfu/ml *S. aureus* (E31290; ■), incubated for 3h. and assayed for plasma TNF-α as described in the Methods section Each point and vertical line represent mean and SEM of 4-12 experiments.

Figure 3. Loss of antagonistic activity during incubation in whole blood.

Ten-fold dilutions of E5564 (●) or E5531 (■) were preincubated in human whole blood for the indicated time (0, 3, or 6h) at 37C, and 10 ng/mL LPS (final concentration) was added and the incubations continued for an additional 3h. Plasma TNF-α was measured, and IC50 interpolated as described in the Materials and Methods section. The differences in IC_{50} between E5564 and E5531 were significant ($p<0.05$) at each time point (Student's T test).

Figure 4. Effects of E5564 on TNF production in BCG primed animals.

The indicated dose of E5564 or vehicle was injected intravenously into mice, guinea pigs and rats (n=5) along with 100, 1000, and 3 μg/kg of LPS, respectively. One hour after administration, blood was collected and TNF-α levels measured. *$P < 0.05$ vs. control group by one-way ANOVA followed by Dunnett's multiple comparison.

Figure 5. The effects of E5564 on mortality induced by *E. coli* LPS in BCG-primed mice.

Vehicle (○) or E5564; 6.25 μg/kg (◆), 12.5 μg/kg (▼), 25 μg/kg (▲), 50 μg/kg (■), or 100 μg/kg (●), was injected intravenously into mice (n=10) along with 100 μg/kg of LPS. Lethality

was observed during 72h. *P < 0.05 vs. control group by Dunnett's multiple comparison test. Incidence of mortality was monitored for 72 hours.

Figure 6. The effects of E5564 on mortality induced by *E. coli* infection in BCG-primed mice. A suspension of *E. coli* (3.23×10^7 cfu/animal) was injected intraperitoneally into BCG primed mice. One hour after inoculation, vehicle (●), 30 mg/kg latamoxef (◆), or 5 mg/kg E5564, alone (■) or with latamoxef (▲) was injected intravenously into mice. The incidence of death was recorded at 12, 24, 48, and 72 hours after administration. Results are expressed as percent mortality of the total number of animals in each group (n=20). *P < 0.05 vs. the other three groups by Fisher's exact test followed by Tukey's multiple comparison test.

Figure 7. E5564 inhibits LPS-induced TLR4-mediated NF-κB reporter activity. HEK293 cells stably transfected with TLR4, MD2, and a luciferase reporter gene driven by an NFκB-dependent promoter, were incubated with the indicated concentrations of E5564 with 100 ng/ml LPS and 10 nM sCD14 for 18-20 h. The next day, luciferase activity was quantified as described in Methods. This experiment is representative of three and each point represents the mean ± SD, n=3.

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Tables

Table 1. E5564 antagonism of LPS- induced cytokine generation in human blood.

Cytokine	Mean and (Minimum/Maximum) Response (pg/ml)	Time of Maximal Response	IC_{50} for E5564 Antagonism (nM)	[E5564] for 100% inhibition (nM)
TNF-α	1142 (908-1436)	3	1.6±0.3	10
IL-1β	851 (731-1078)	24	<1	10
IL-6	13974 (7932-22207)	24	1-2	100
IL-8	22719 (2181- 36098)	24	13	~100
IL-10	534 (458-595)	24	~1	10

Human blood was incubated for 3, 4, 6, 9, or 24 hours in the presence of 10 ng/mL LPS plus 0, 1, 10, or 100 nM E5564 at 37° C as described in figure 1. After the incubation, the blood was centrifuged, and the resulting plasma supernatant samples were assayed for the indicated cytokine. Values are presented as the mean and SE for three assays. The IC_{50} inhibition values presented are the highest mean values derived by calculating the IC_{50} for each cytokine at each time point shown in Figure 1 (excluding the 24-hour values for TNF-α). .. Antagonism of each cytokine was determined from three or more assays done with triplicate determinations.

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Table 2. E5564 inhibition of TNF-α induced by LPS from various strains of bacteria, dead bacteria, and Lipid A.

Agonist Added: Strain of LPS[1]	**Amount Added (ng/ml)**	**TNF-α released (pg/mL) Mean ± SE**[2]	**Antagonism by E5564 Average IC_{50} (nM)**
Klebsiella pneumoniae	10	2868 ± 104	8.5 ± 5.0
Psuedomonas aeruginosa	10	2027 ± 185	1.0 ± 0.21
Salmonella minnesota	10	2793 ± 99	12.4 ± 5.1
Salmonella enteritidis	1	2279 ± 184	2.6 ± 0.47
Salmonella typhimurium	10	3091 ± 182	9.4 ± 6.7
Serratia marcescens	10	3128 ± 91	10.3 ± 6.2
Salmonella minnesota R595	10	1578 ± 284	7.6 ± 2.9
Escherichia coli	10	1142 ± 155	1.6 ± 0.3
Whole Bacteria[3]			
Enterobacter aerogenes (ATCC)	100	2165 ± 299	1.5 ± 0.7
Enterobacter aerogenes (clinical isolate)	100	2558 ± 389	1.2 ± 0.5
Whole *Eschericia coli*	100	3172± 413	0.65 ± 0.32
Lipid A			
E. coli	10	2500± 294	1.2 ± 0.7

[1]LPS from *Vibrio cholerae* Inaba 569B and *Bordetella pertussis* 165 were also analyzed, however, they were only weakly active at stimulating release of TNF-α from whole blood and stimulation was highly variable. E5564 inhibited this weak stimulation with IC_{50} values of 1 nM or less for both strains.

[2]Each value represents the mean and standard error of triplicate determinations obtained from

three experiments.

[3] ng/ml of whole bacteria from lyophilized powder. Refer to Materials and Methods.

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Table 3. E5564 inhibition of TNF-α and/or IL-6 induced by LPS in peritoneal macrophages and whole blood from mice, rats and guinea pigs.

	Cytokine assayed:			
	TNF-α		IL-6	
Assay:	TNF-α Induced (pg/mL)	E5564 IC_{50} (nM)	IL-6 Induced (ng/mL)	E5564 IC_{50} (nM)
Mouse peritoneal macrophages	3315 ± 318	20.4 ± 12.5	5.0 ± 0.53	16.6 ± 6.7
Mouse blood	NT	NT	13.0 ± 0.18	20.2 ± 7.0
Rat peritoneal macrophages	2867 ± 326	7 ± 5.6	93 ± 99 (range 23-163)	16.2 ± 17.5 (range 3.9-28.6)
Rat blood	2241 ± 335	136 ± 61	55.8 ± 12	~2400
Guinea pig Macrophages	1897 ± 348	0.3 ± 0.15	3.0 ± 0.43	0.5 ± 0.3

Cells or blood prepared as described in the Materials and Methods section were stimulated with 10 ng/ml LPS plus a range of doses of E5564 for two or three hours. Supernatant or plasma samples were assayed for the indicated cytokines. Most values were determined from triplicate incubations done three times, except rat peritoneal macrophages (n = 2). Basal induction of cytokine (cytokine values measured after incubation in the absence of LPS) was 4% or less of values from the LPS-stimulated samples in all cases.

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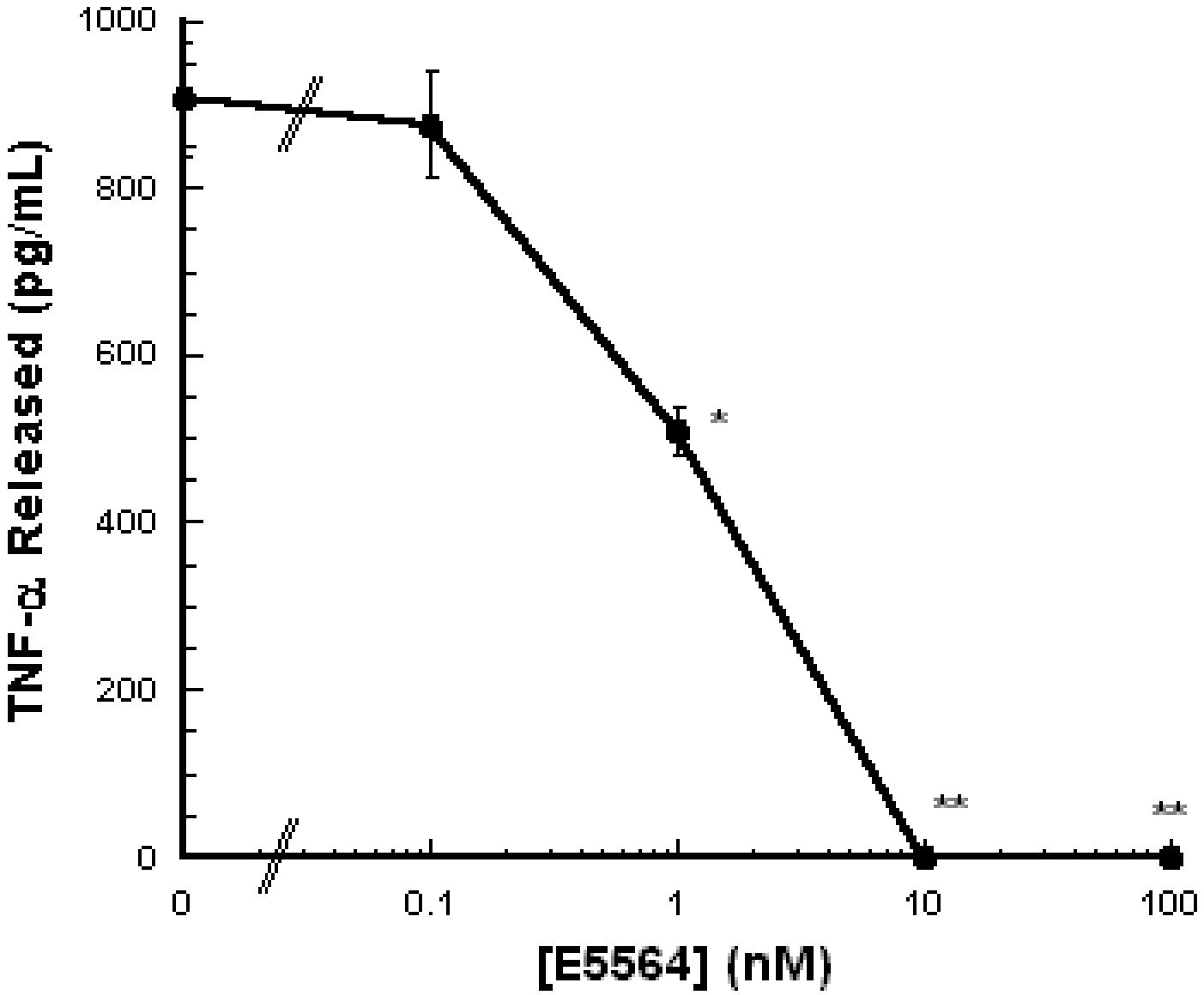
Table 4. Activity of E5564 after different conditions of preincubation and dissociation incubation with cells

Time of Dissociation (hours)	Activity (IC_{50}) of E5564 (in nM)after incubation with RAW 264.7 cells preincubated in the absence (pre-) or presence (pre+) of serum followed by dissociation incubation in the absence (post -) or presence (post +) of serum			
	pre-/post-	pre+/post-	pre-/post+	pre+/post+
0	0.8 ± 0.1	1.1 ± 0	0.8 ± 0.1	1.1± 0
0.5	0.7± 0.1	2.5	1.1	5.1
2	*	*	25	24.5

Raw 264.7 cells were incubated for one hour in the presence or absence of 10% fetal bovine serum and in the absence or presence of E5564 at 100, 33, 11, 3.3, 1.1 nM. Cells were then washed three times in serum free- medium, incubated in the same media with or without added fetal calf serum (10%) for the indicated period of time, washed again, and assayed for LPS - mediated generation of TNF-α as described in the Methods and Materials section.

*Poorly reproducible. Incubation in serum-free media for an extended period of time combined with extensive washing resulted in loss of adherent cells and cell death. Cytokine release was low and variable.


1000
800
600
400
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TNF-α Released (pg/mL)
0
0.1
1
10
100
[E5564] (nM)
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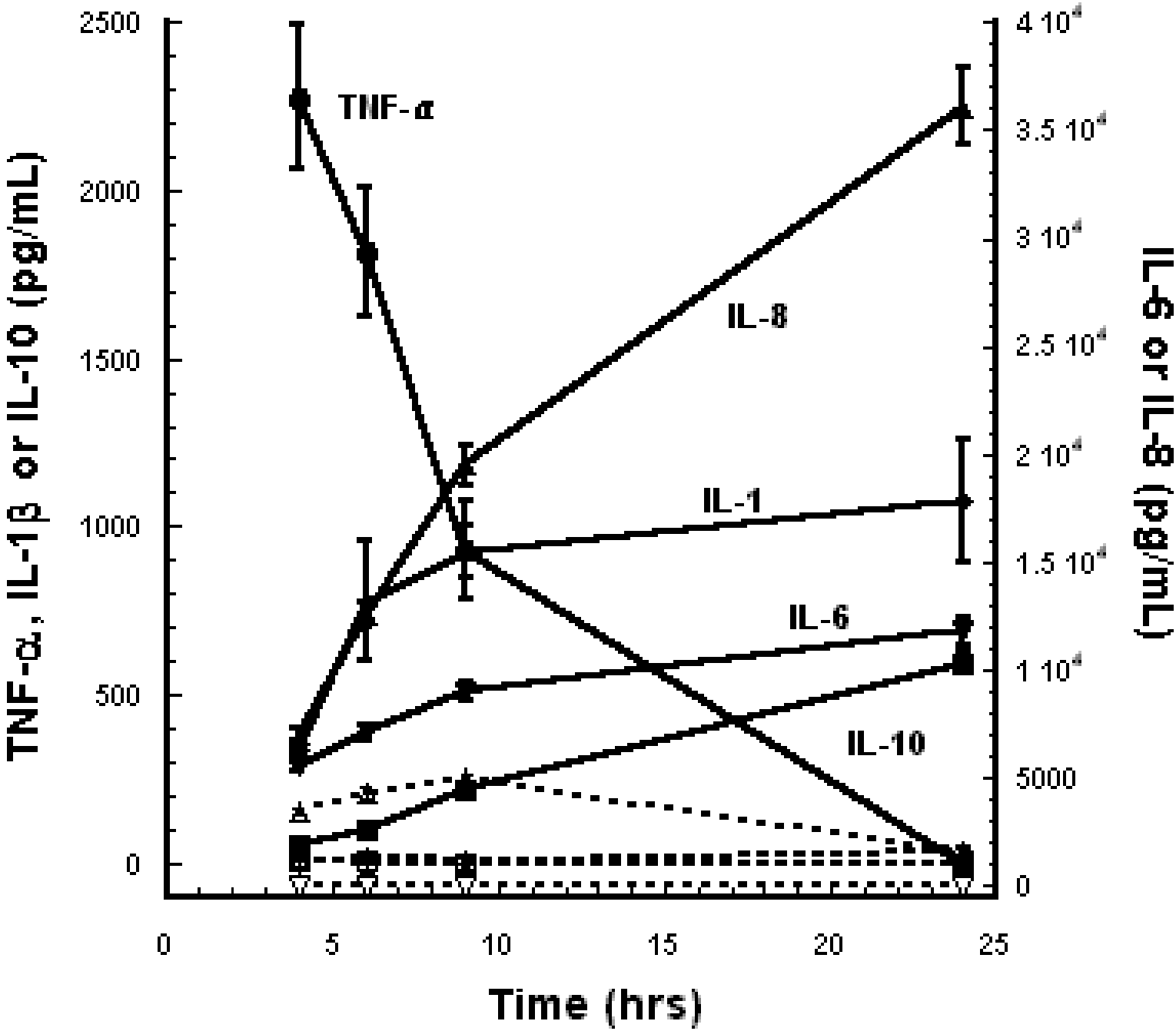
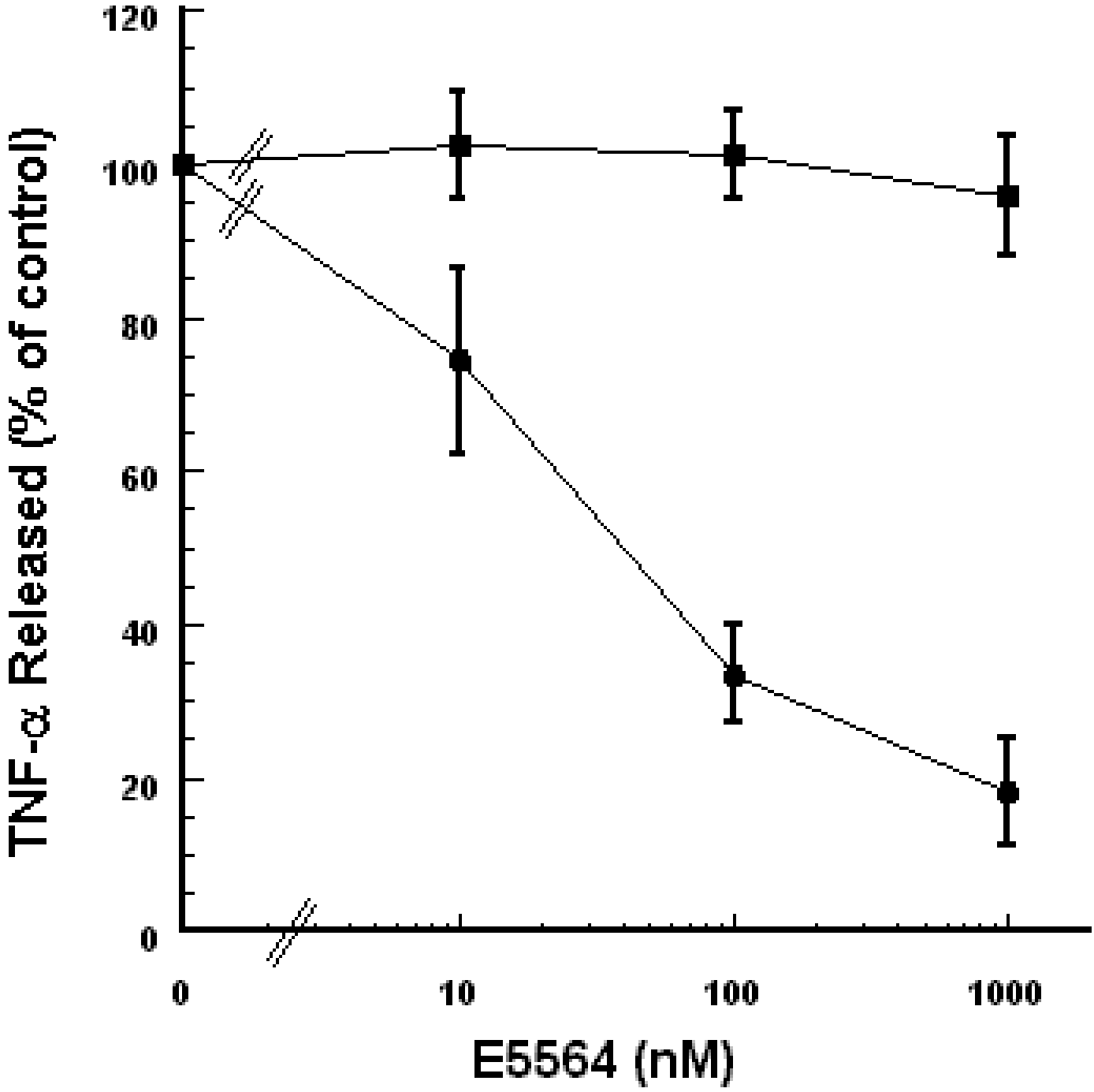
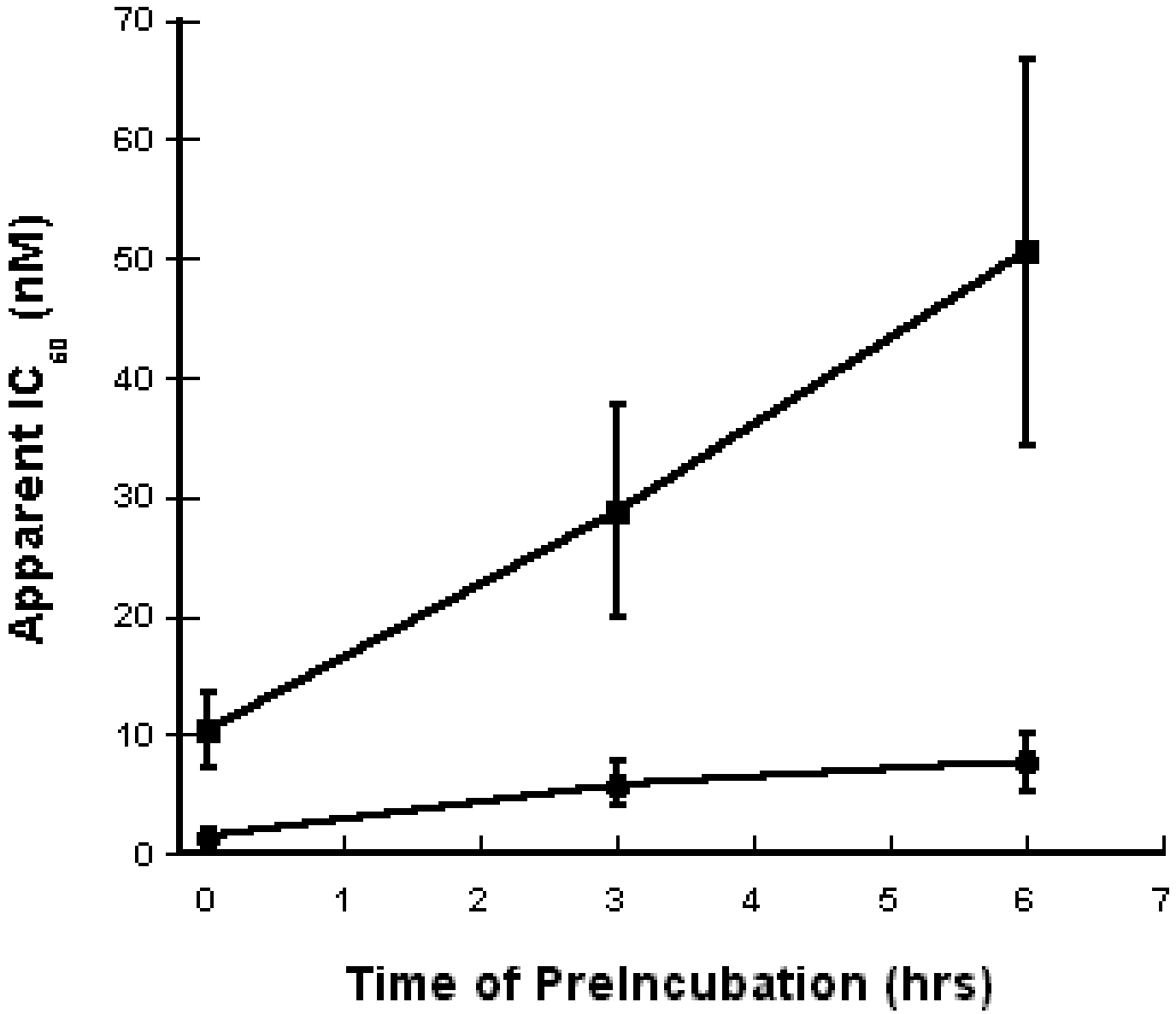
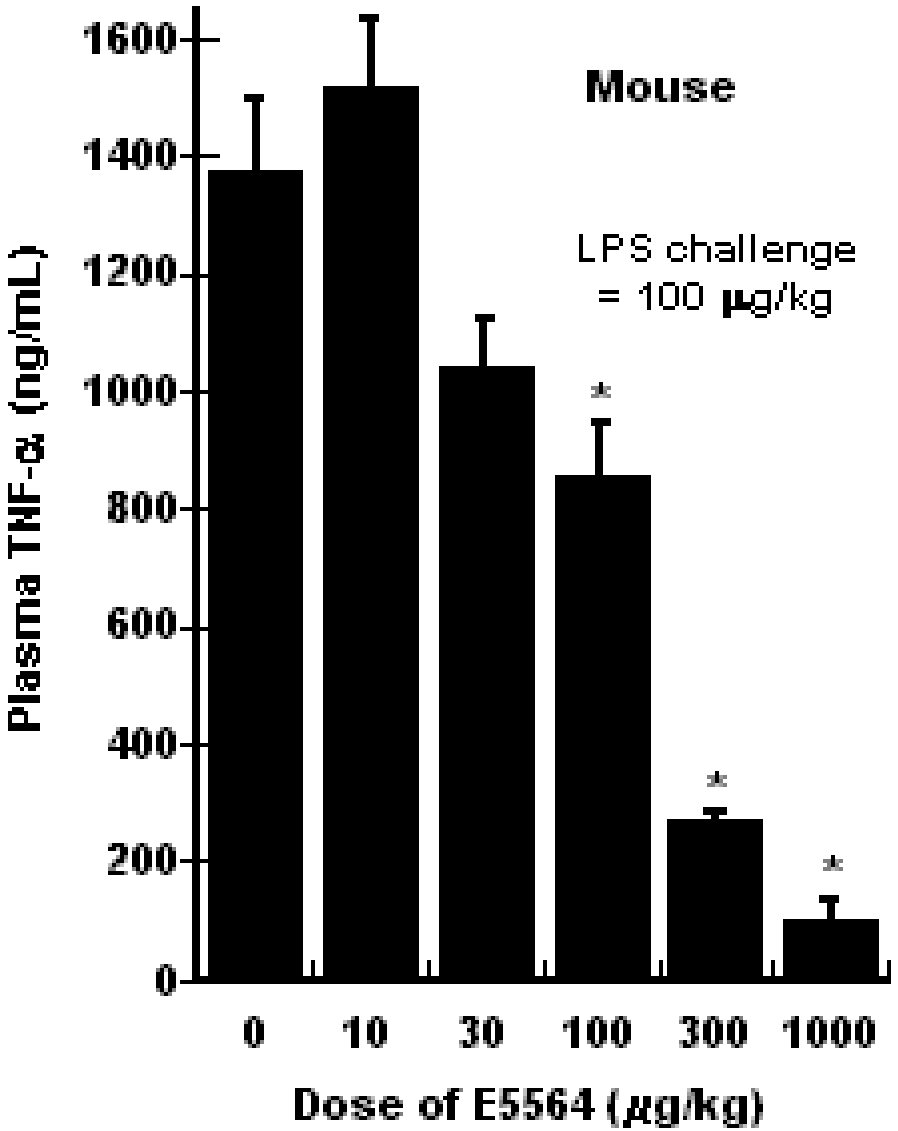
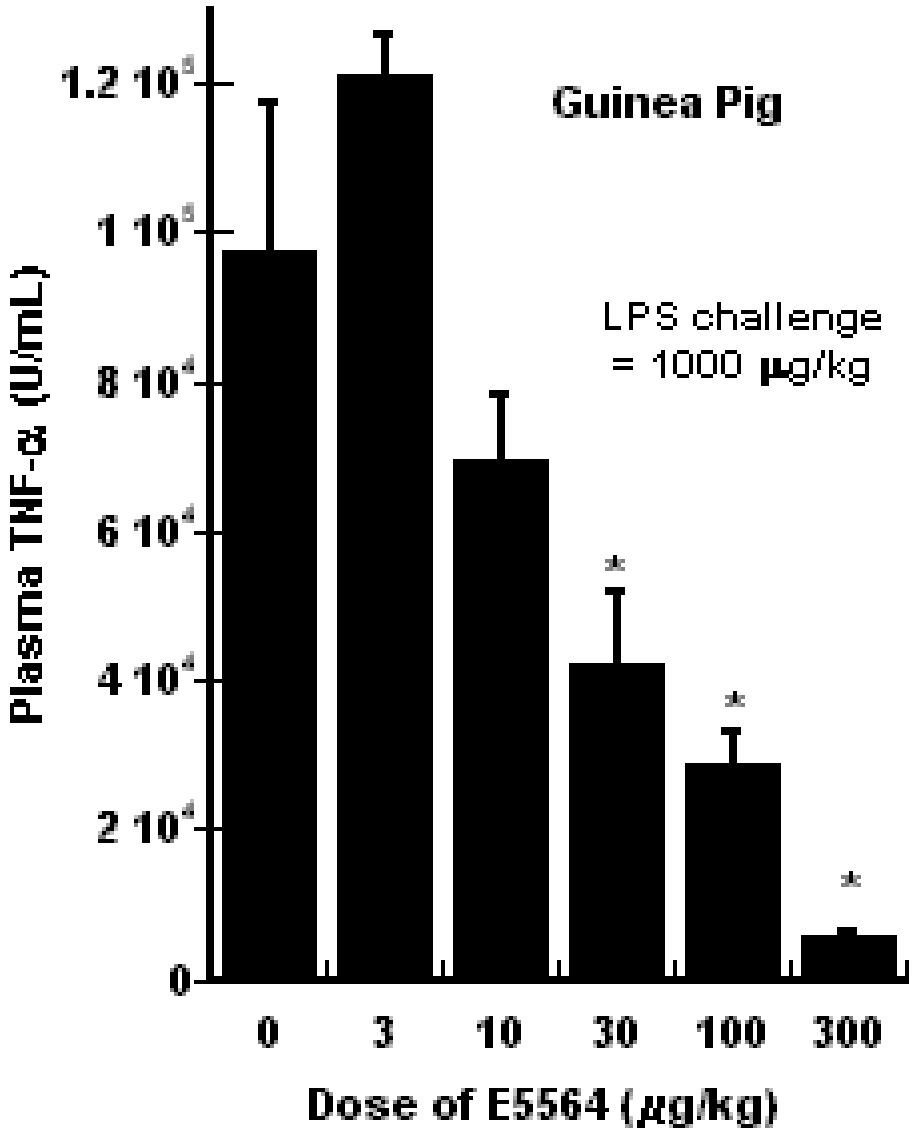
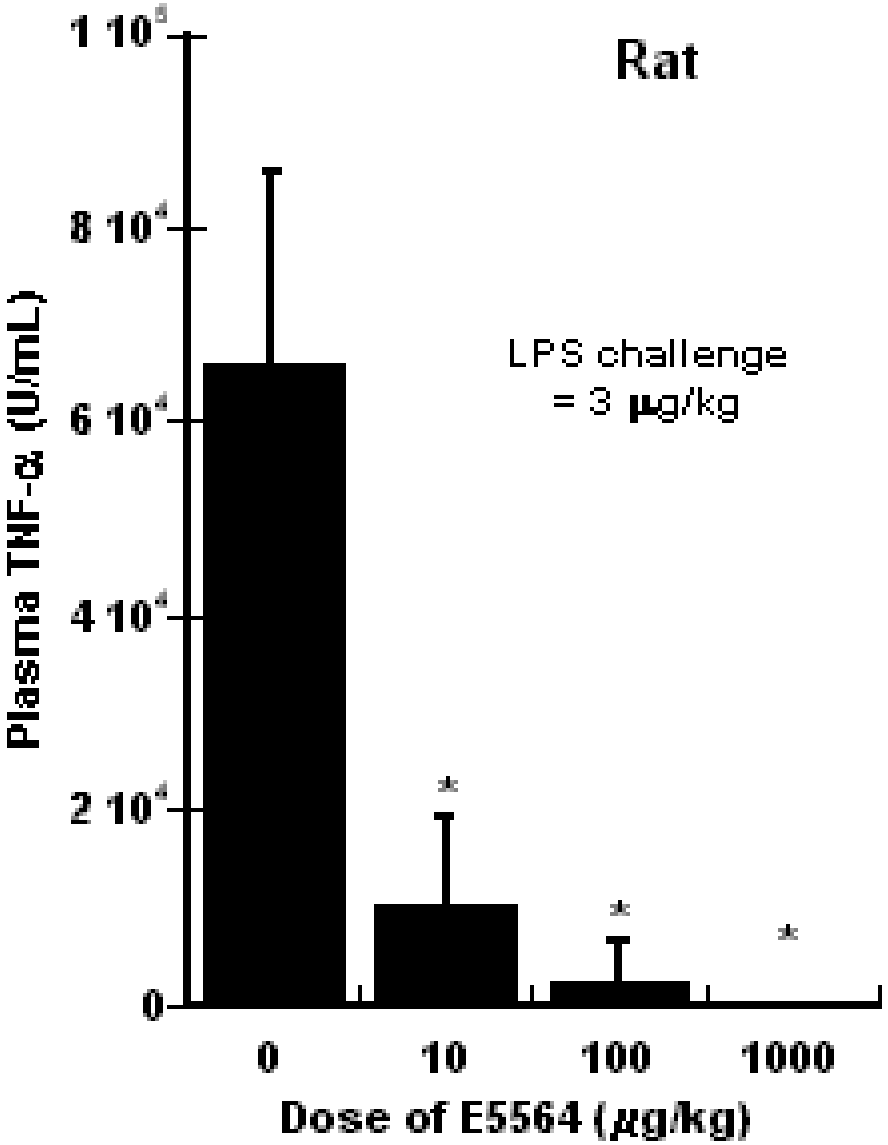

TNF-α
IL-8
IL-1
IL-6
IL-10
TNF-α, IL-1β or IL-10 (pg/mL)
IL-6 or IL-8 (pg/mL)
Time (hrs)
2500
2000
1500
1000
500
0
4 10^4
3.5 10^4
3 10^4
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
0
5
10
15
20
25

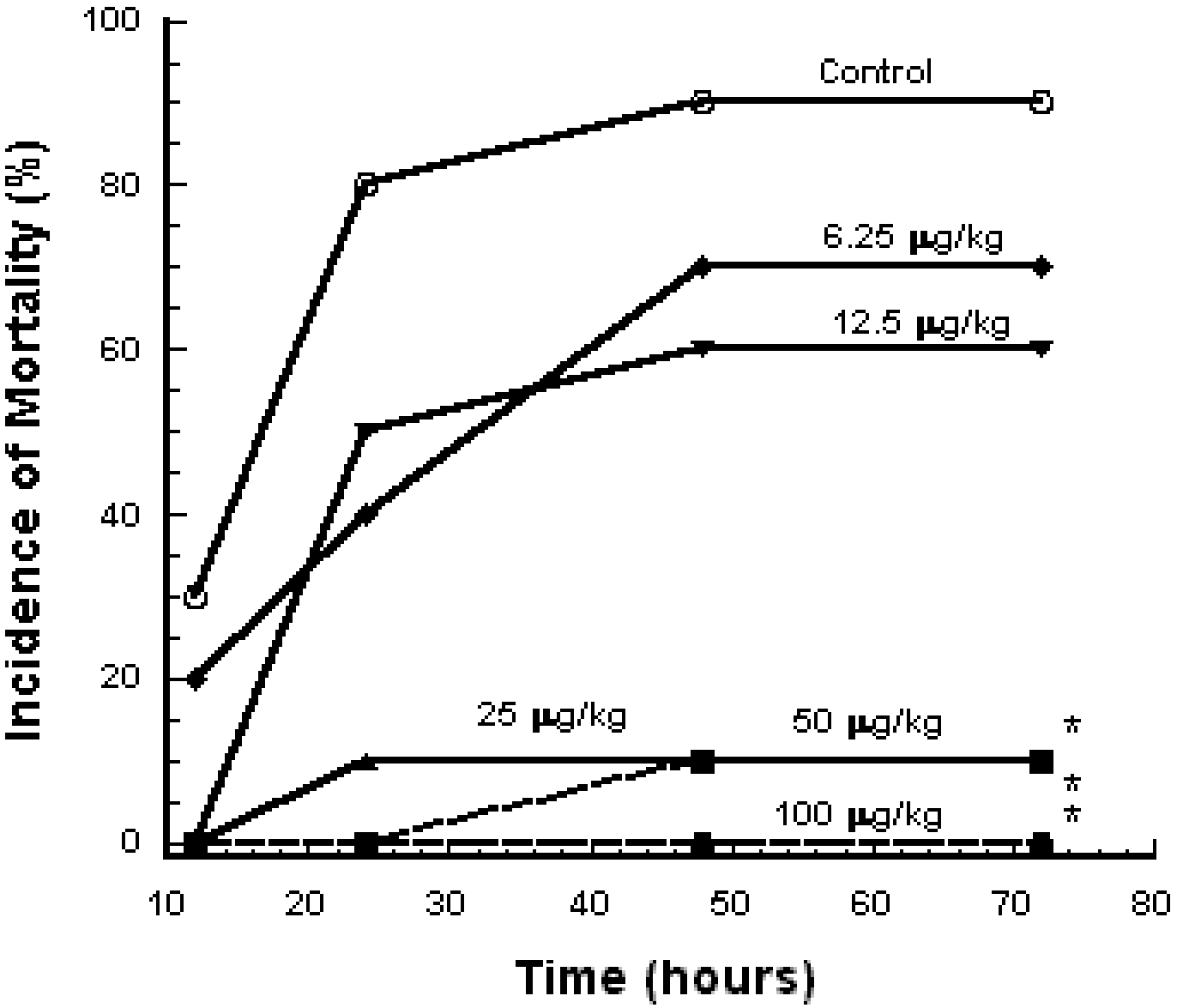

TNF-α Released (% of control)
120
100
80
60
40
20
0
0
10
100
1000
E5564 (nM)

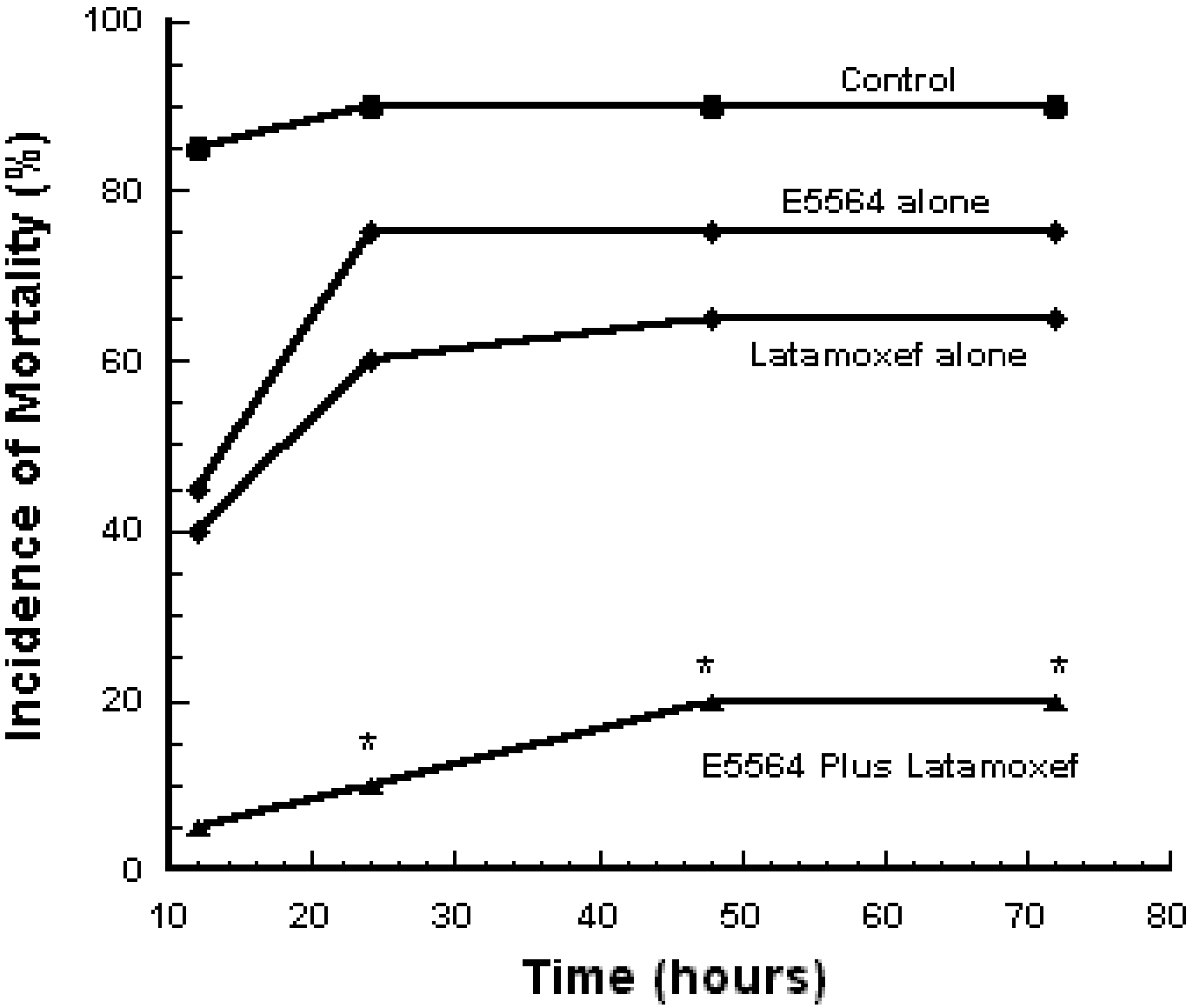

70
60
50
40
30
20
10
0
Apparent IC60 (nM)
0
1
2
3
4
5
6
7
Time of PreIncubation (hrs)

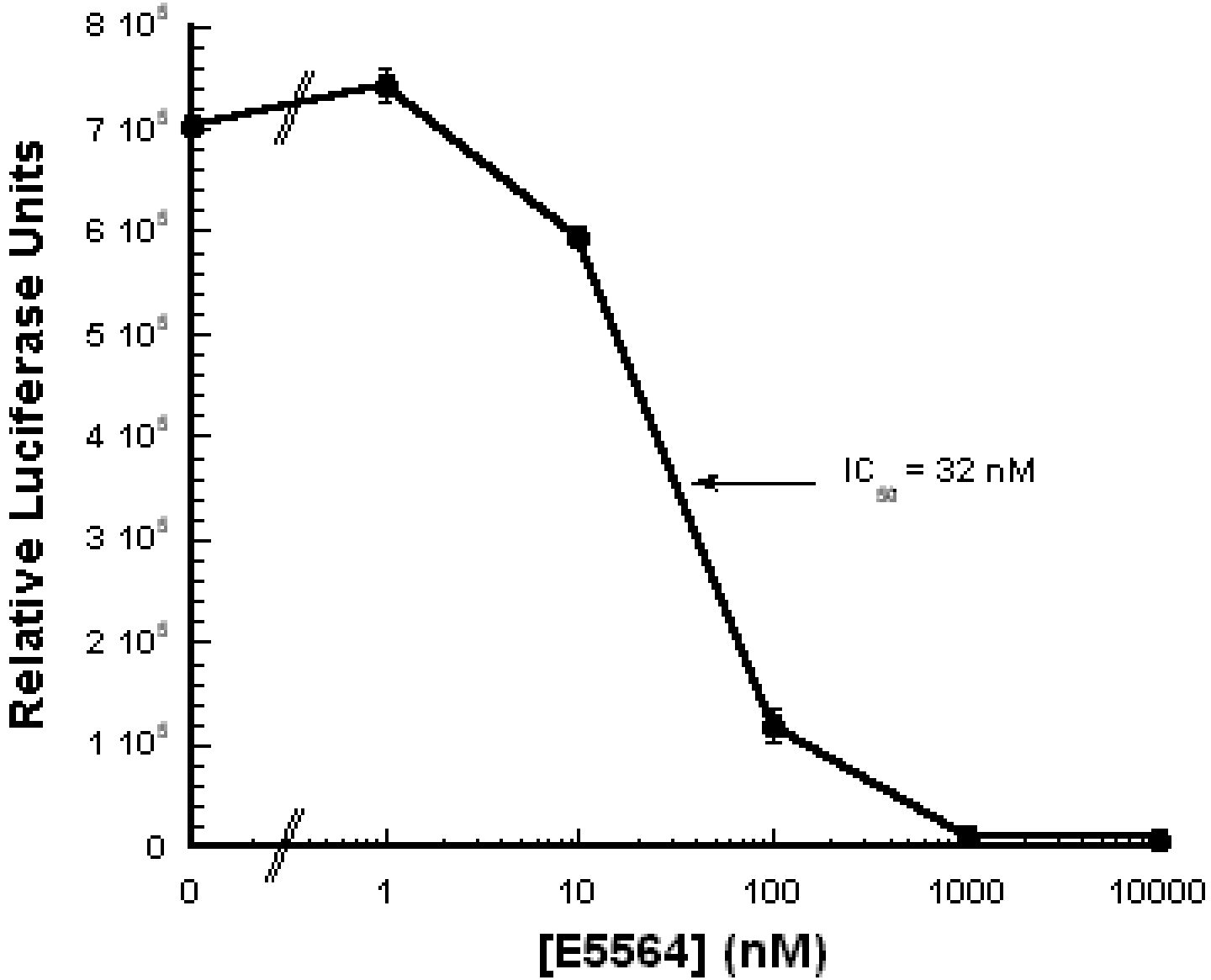

Mouse
LPS challenge
= 100 μg/kg
Plasma TNF-α (ng/mL)
1600
1400
1200
1000
800
600
400
200
0
*
*
*
0
10
30
100
300
1000
Dose of E5564 (μg/kg)


Guinea Pig
LPS challenge
= 1000 µg/kg
Plasma TNF-α (U/mL)
1.2 10⁵
1 10⁵
8 10⁴
6 10⁴
4 10⁴
2 10⁴
0
*
*
*
0
3
10
30
100
300
Dose of E5564 (µg/kg)


Rat
LPS challenge
= 3 µg/kg
Plasma TNF-α (U/mL)
1 10^5
8 10^4
6 10^4
4 10^4
2 10^4
0
*
*
*
0
10
100
1000
Dose of E5564 (µg/kg)


Incidence of Mortality (%)
100
80
60
40
20
0
10
20
30
40
50
60
70
80
Time (hours)
Control
6.25 µg/kg
12.5 µg/kg
25 µg/kg
50 µg/kg
100 µg/kg
*
*
*


Incidence of Mortality (%)
100
80
60
40
20
0
10
20
30
40
50
60
70
80
Time (hours)
Control
E5564 alone
Latamoxef alone
E5564 Plus Latamoxef
*
*
*


Relative Luciferase Units
8 10^5
7 10^5
6 10^5
5 10^5
4 10^5
3 10^5
2 10^5
1 10^5
0
0
1
10
100
1000
10000
[E5564] (nM)
IC50 = 32 nM